

lamellipodiae and at cell cell contact sites. Cell motility and cell paths did not increase ( $19,7-97,2 \mu\text{m}$ ;  $5,0-24,3 \mu\text{m/h}$ ; Fig. 27 + 28).

Taken together, our results demonstrate that the localization of E-cadherin after application of Tyrphostin AG1478 or EGF depends on the mutation status of E-cadherin. *wt*-EcadEGFP which is normally localized at cell cell contact sites, is found in the cytoplasm, the perinuclear region and in lamellipodia after application of EGF. In contrast, the EGFR inhibitor Tyrphostin AG1478 has no influence on the localization of *wt*-EcadEGFP. *p8*-EcadEGFP is normally localized at cell edges, in lamellipodiae and at transiently formed cell cell contact sites. EGF had no influence on *p8*-EcadEGFP localization, while Tyrphostin AG1478 caused that *p8*-EcadEGFP was localized at cell cell contacts and perinuclear.

### References of Example VIII

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**Example IX: Epidermal Growth Factor Receptor Immunohistochemical Reactivity at the Invasion Front Correlates with Poor Survival in Gastric Adenocarcinoma from Mexican Patients.**

The aim of this example was to determine epidermal growth factor receptor (EGFR) expression in gastric adenocarcinoma by standardized immunohistochemistry using an EGFR detection system and to correlate EGFR expression with clinical features and patient survival.

For this purpose, EGFR expression was investigated in paraffin sections of resection specimens of 89 gastric carcinomas. Membrane staining of EGFR was evaluated in the neoplastic cells and graded using a semiquantitative score (0-3+). Staining of neoplastic cells was negative in 47 cases (52.8 %), weak in 17 tumors (19.1 %, score 1+), moderate in 16 adenocarcinomas (18.0 %, score 2+) and strong in 9 neoplasms (10.1 %, score 3+). EGFR reactivity was very heterogeneous, frequently showing completely negative up to 3+ positive areas. A correlation was found between EGFR reactivity score and distant metastases ( $p=0.002$ ) or clinical stage ( $p=0.033$ ), but not between EGFR score and histotype, tumor invasion, perigastric lymph node status or residual disease. EGFR score 0/1+ was significantly associated with an increase in patient survival, when compared to score 2+/3+ ( $p=0.0006$ ). The presence of EGFR reactive cells in muscle layer and subserosa was associated with a decrease in patient survival ( $p=0.0004$ ). Cox regression analysis revealed that the prognosis was associated with the EGFR reactivity score, EGFR reactive neoplastic cells in mucosa ( $p=0.019$ ), muscularis or subserosa ( $p=0.001$ ), submucosa, muscularis or subserosa (0.002), distant metastases ( $p=0.0001$ ) and residual disease ( $p=0.012$ ) in an univariate analysis. A multivariate analysis revealed that EGFR positive cells in muscularis or subserosa ( $p=0.004$ ), distant metastases ( $p=0.016$ ) and residual disease were significantly correlated with a decrease in survival (0.012). Accordingly, it can be shown that EGFR reactivity at the deep tumor invasion front is correlated with poor survival in gastric adenocarcinoma.

## **Materials and Methods for this example**

### **Patient Selection**

Patients with total gastrectomy operated with the diagnosis of adenocarcinoma since 1982 to 2001 in the Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran, with available clinical information and follow-up were considered. The clinical variables obtained from the charts were age at diagnosis, gender, survival time and cause of death. A blinded review of all the slides was made by pathologists and a diagnosis according to Laurén's classification of gastric adenocarcinoma was made (Laurén, Acta Pathol. Microbiol. Scand. 64

(1965), 31-49). Mestizo Mexican patients with available paraffin material and a morphologic diagnosis of poorly differentiated intestinal, mixed or diffuse-type adenocarcinoma in which UICC staging criteria (Spiessl, (1992), loc. cit.) could be reproduced, were included.

### **EGFR Immunohistochemistry**

A hematoxylin/eosin stained section was obtained and reviewed for morphologic confirmation and two consecutive sections were mounted on charged slides for immunohistochemistry. Immunostainings for EGFR were performed using the Dako EGFRpharmDx<sup>TM</sup> assay detection system (Dako Corporation, Carpinteria, CA) which recognizes a 170 kDa transmembrane receptor encoded by the human HER1 gene. The manual staining protocol was precisely followed, and no substitutions were made. After dewaxing in fresh xylene, 100 % ethanol, 95 % ethanol and 70 % ethanol (4 baths each), the slides were placed in a humid chamber for proteolytic digestion with proteinase K solution (100 µl for 5 min), and quenching of endogenous peroxidase for 5 min. The primary antibody was incubated for 30 min followed by 30 min incubation with labelled polymer, and DAB localization of the positive cells. Counterstain was made with hematoxylin followed by 10 slide dips in a bath containing 37 mmol/l ammonia water. In every run control slides were included which were provided to validate the performance of the reagents of the Dako EGFRpharmDx<sup>TM</sup> assay detection kit. The control slides contained sections of pelleted, formalin-fixed, paraffin-embedded cell line HT-29 with a moderate level of EGFR protein expression (positive control, IHC staining score of the cell pellet is  $2.5 \pm 0.5$ ) and of the EGFR negative CAMA-1 cell line (negative control, score 0).

### **EGFR reactivity evaluation**

Membrane staining was evaluated in the neoplastic cells and quantified and graded as recommended in the detection kit:

0 score      No staining observed, or membrane staining in <10% neoplastic cells. Negative.

1+ score	Weak complete and/or incomplete membrane staining in >10% neoplastic cells. Positive.
2+ score	Moderate complete and/or incomplete membrane staining in >10% neoplastic cells. Positive.
3+ score	Strong complete and/or incomplete membrane staining in >10% neoplastic cells. Positive.

Localization and intensity of reactivity was evaluated for mucosa, submucosa and deeper zones (muscle layer and subserosa). Statistical analyses were performed using Fisher's exact, K and  $X^2$  tests when appropriate. Kaplan-Meier survival time analysis was used to correlate EGFR reactivity, localization of positive cells (surface or deep), pT, pN and pM status with clinical evolution. Cox regression analysis was performed correlating EGFR reactivity, localization of positive cells, and stage with prognosis. A two sided p value less than 0.05 was considered to be statistically significant.

## RESULTS

### Clinicopathological Features

The clinicopathological features of 89 Mestizo Mexican patients with gastric cancer are shown in appended Table 1. The mean and median of the patient ages at the time of diagnosis were 57.8 or 60.0 years, respectively, with a range of 14-86 years and a standard deviation of 15.2 years. 44 patients (49.4 %) were female, 45 patients (50.6 %) were male. The gastric cancer histotype was classified according to Laurén: 36 cases (40.4 %) were of poorly differentiated intestinal type, 49 tumor samples (55.1 %) were of diffuse type and 4 cases (4.5 %) were of mixed type, containing both intestinal and diffuse components. The stages (UICC) were IB in 1 patient (1.1 %), II in 25 patients (28.1 %), IIIA in 20 patients (22.5 %), IIIB in 14 cases (15.7 %) and IV in 29 cases (32.6 %). The residual disease status was R0 in 69 cases (77.5 %) and R1 in 20 cases (22.5 %).

### **EGFR Score of Reactivity**

89 slides from gastric carcinomas were stained with the EGFR immunohistochemical detection system. Membrane staining was evaluated in the neoplastic cells and quantified and graded as recommended in the detection kit (appended Table 2). 47 cases (52.8 %) were negative or reactive in <10 % of neoplastic cells (score 0). Complete and/or incomplete membrane staining in >10 % of neoplastic cells was weak in 17 tumors (19.1 %, score 1+), moderate in 16 adenocarcinomas (18.0 %, score 2+, Fig. 37) and strong in 9 neoplasms (10.1 %, score 3+). The percentage of EGFR reactive cells per case was also evaluated, without considering the staining intensity. 26 cases (29.2 %) were completely EGFR negative. 21 cases (23.6 %) showed reactivity in <10 % of neoplastic cells, 30 cases (33.7 %) were reactive in 10-50 % of tumor cells and 12 cases (13.5 %) were positive in >50 % of neoplastic cells. Nerve and muscle cells served as reactive internal control. Normal gastric mucosa showed no EGFR staining. EGFR reactivity frequently showed a striking variability in the tumor tissue. In some cases, only few tumor cells were highly reactive (score 3+), while the rest of the tumor showed low reactivity or complete absence of EGFR expression.

### **EGFR Score and its Correlation with Clinicopathological Features and Morphology**

The EGFR score of reactivity was correlated with clinicopathological features and morphology (appended Table 3). EGFR reactivity score 2+/3+ was present in 9/36 intestinal type, in 1/4 mixed type and in 15/49 diffuse type gastric carcinomas. There was no statistically significant association between EGFR score and histotype according to Laurén ( $p=0.201$ ). In 0/8 cases with tumor invasion stage pT 2 and in 25 cases with stage pT 3-4, EGFR reactivity score 2+/3+ was detectable. EGFR score and depth of tumor invasion were not significantly correlated ( $p=0.304$ ). 3/21 cases with perigastric lymph node status pN0 and 22/68 cases with status pN1-2 showed EGFR score 2+/3+. There was no correlation between EGFR score and perigastric lymph node status ( $p=0.313$ ). EGFR reactivity score 2+/3+ was present in 16/71 cases without distant metastases and in 9/18 cases with metastases. The association between distant

metastases and EGFR reactivity score was statistically significant ( $p=0.002$ ). In 17/69 cases with residual disease status R0 and in 8/20 cases with status R1 EGFR score 2+/3+ was present. There was no significant association between EGFR score and residual disease ( $p=0.406$ ). EGFR reactivity score 2+/3+ was detectable in 2/26 cases with clinical stage I-II and in 22 cases with stage III-IV. The correlation between EGFR score and clinical stage was statistically significant ( $p=0.033$ ).

### **Influence of EGFR Score and Percentage of EGFR Reactive Neoplastic Cells on Survival**

Kaplan Meier survival time analysis was used to correlate EGFR score and percentage of reactive neoplastic cells with patient survival. Survival time of patients with EGFR scores 0/1+ was significantly increased when compared with EGFR score 2+/3+ ( $p=0.0006$ , Figure 36). The log rank test statistical analysis indicates a global  $p$  value 0.0083. When the percentage of EGFR reactive cells was correlated with patient survival, no EGFR reactivity or reactivity in <10% cells resulted in increased patient survival when compared with EGFR reactivity in 10-50 % or >50 % cells. This trend was observable, although the result did not reach statistical significance (global  $p$  value 0.0688). The mean and median of the overall patient follow-up were 21.3 month or 12.0 month, respectively, with a range of 1-173 months and a standard deviation of 28.8 month.

### **Distribution of EGFR Reactive Neoplastic Cells and Association of EGFR Reactivity at the Invasion Front with Survival**

Localization and intensity of reactivity was evaluated for mucosa, submucosa and deeper zones (muscle layer and subserosa). The presence of EGFR reactive cells at the invasion front was significantly associated with a decrease in patient survival (global  $p$  value 0.0004, Figure 38).

Cox regression analysis was performed to correlate EGFR reactivity score, percentage and localization of positive cells, stage, distant metastases and residual disease status with prognosis (appended Table 4, univariate all patients). EGFR reactivity score was associated with the length of survival ( $p=0.003$ ). In



contrast, the percentage of EGFR positive cells was not correlated with patient survival ( $p=0.071$ ), although a trend was detectable. Furthermore, a significant association with survival was observed for positive neoplastic cells in mucosa ( $p=0.019$ ), muscularis or subserosa ( $p=0.001$ ), submucosa, muscularis or subserosa ( $p=0.002$ ), distant metastases ( $p=0.001$ ) and residual disease ( $p=0.012$ ). A multivariate analysis revealed that EGFR positive cells in muscularis or subserosa ( $p=0.004$ ), distant metastases ( $p=0.016$ ) and residual disease ( $p=0.039$ ) were significantly correlated with a decrease in survival ( appended Table 4).

Kaplan Meier survival time analysis was used to correlate residual disease status with patient survival. Survival time of patients with status R0 was significantly increased when compared with status R1 (global  $p$  value 0.0028, Figure 5).

### **EGFR as Prognostic Marker in Gastric Cancer**

Data reported in this example finding that EGFR reactivity score has been identified as a prognostic indicator in gastric cancer is in accordance with several studies which have demonstrated that EGFR expression correlates with poor prognosis (Nicholson, Eur. J. Cancer 27 Suppl. 4 (2001), 9-15). Recently, the relationship between EGFR expression and cancer prognosis was investigated based on the analysis of literature data of more than 200 studies published between 1985 and 2000 (Nicholson (2001), loc. cit.). It was found that EGFR expression was a strong prognostic indicator in cancers of the head and neck, ovary, cervix, bladder, and esophagus, and that EGFR expression correlated with reduced recurrence-free and overall survival in 70% of studies included in the literature search. In gastric, breast, endometrial and colorectal cancers EGFR expression was associated with poor survival in 52% of the included studies, while in non-small-cell-lung cancer only 30% of studies showed such a correlation between EGFR expression and survival. For gastric cancer, co-expression of EGFR and its ligands EGF or TGF- $\alpha$  was found to be correlated with a decrease of survival or the relapse-free survival interval (Yasui, Int. J. Cancer 41 (1988), 211-217; Yonemura, Oncology 49 (1992), 157-161; Tokunaga, Cancer 75 (1995), 1418-1425). Amplification (Tsugawa, Oncology 55 (1998), 475-481; Hirono,

Oncology 52 (1995), 182-188) or expression of EGFR (Yasui (1988), loc. cit.) was correlated with advanced clinical stage and the presence of lymph node metastasis (Yasui, Cancer Res. 48 (1988), 137-141; Iida, Oncology 52 (1995), 189-195).

### **EGFR Positivity at the Deep Invasion Front**

In the present study, the presence and staining intensity of EGFR reactive cells were evaluated in mucosa, submucosa and at the deep invasion front in muscle layer and subserosa after exclusion of patients with early cancer in mucosa and submucosa. The localization of EGFR reactive cells in muscle layer and subserosa was associated with a decrease in patient survival which indicates that EGFR positivity at the deep invasion front is critical in determining the patient's outcome. In a recent study using the same technique in colonic adenocarcinoma, positivity at the invasion front also showed the strongest correlation with survival duration as well as with EGFR positivity of lymph node and liver metastases (Goldstein, Cancer 92 (2001), 1331-1346). Increased EGFR expression at the most invasive parts of carcinomas has also been reported for oral squamous cell carcinomas (Bankfalvi, J. Pathol. 198 (2002), 343-351). These data support the hypothesis that the invasive front of carcinomas is the most critical area for prognostication (Goldstein (2001), loc. cit.; Bryne, Anticancer Res. 18 (1998), 4757-4764; Bankfalvi, J. Oral Pathol. Med. 29 (2000), 291-298).

### **Heterogeneity of EGFR Expression**

EGFR reactivity showed a marked intratumoral heterogeneity, frequently showing a range of variations of completely negative up to 3+ positive neoplastic cells within an individual case. EGFR staining heterogeneity was also observed for colonic adenocarcinoma (Goldstein (2001), loc. cit.). These observations argue for an up-regulation of EGFR expression in later stages of tumor progression. Different mechanisms, like autocrine stimulation by growth factors, genetic instability or transcriptional dysregulation may be considered. With regard to anti-EGFR therapy, the impact of EGFR heterogeneity on the therapeutic response

has to be clarified. It may also be of importance in the evaluation of small tumor samples, e. g. pretherapeutic endoscopic samples.

### **Example X: Correlative Analysis of Epidermal Growth Factor Receptor Expression and Immunohistochemical Reactivity with Mutation-specific E-cadherin Antibodies**

#### **Abbreviations used in this example**

CI: confidence intervall; *del 8* E-cadherin, E-cadherin with deletion of exon 8; *del 9* E-cadherin, E-cadherin with deletion of exon 9.

This example X was undertaken to determine epidermal growth factor receptor (EGFR) expression in gastric adenocarcinoma by standardized immunohistochemistry and immunohistochemical reactivity with mutation-specific E-cadherin antibodies recognizing E-cadherin lacking exon 8 (*del 8*) or 9 (*del 9*). EGFR and *del 8* or *del 9* E-cadherin expression were examined in paraffin-embedded resection specimens of 92 gastric carcinomas from Mexican Mestizo patients. The gastric cancer histotype according to Laurén was intestinal type in 37 cases (40.0 %), diffuse type in 51 tumor samples (55.0 %) and mixed type in 4 cases (5.0 %). EGFR expression was investigated using a standardized detection system. Membrane staining of EGFR was evaluated in the tumor cells and graded using a semiquantitative score (0-3+). EGFR expression was observed in 43 patients (47.0 %). Staining of neoplastic cells was weak in 17 tumors (19.5 %, score 1+), moderate in 17 adenocarcinomas (18.5 %, score 2+), strong in 9 neoplasms (10.0 %, score 3+), and negative in 49 cases (53.0 %). In an univariate analysis, EGFR reactivity score 2+/3+ ( $p=0.002$ ) and stage III/IV ( $p=0.02$ ) were significantly associated with prognosis. Multivariate analysis using Cox's proportional hazard model revealed that EGFR reactivity score 2+/3+ ( $p=0.012$ ) and stage III/IV ( $p=0.028$ ) were significantly associated with poor prognosis. *del 8* or *del 9* E-cadherin reactivity in combination with EGFR reactivity and stage further decreases the survival prognosis. Since *del 8* or *del 9* E-cadherin reactivity is predominantly found in diffuse and mixed type gastric carcinoma, further

analysis was performed with 55 cases, after exclusion of intestinal type tumor patients. In an univariate analysis, stage III/IV ( $p=0.007$ ) was significantly associated with prognosis. Multivariate analysis using Cox's proportional hazard model revealed that stage III/IV ( $p=0.005$ ) was significantly associated with poor prognosis. *del 8* or *del 9* E-cadherin reactivity in combination with stage further decreases the survival prognosis.

**Conclusion:** Our data indicate that in combination with EGFR score 2+/3+ and stage III/IV, *del 8* or *del 9* E-cadherin reactivity contributes to poor prognosis in gastric carcinoma. When only diffuse and mixed type gastric carcinomas are considered, stage III/IV is the most important prognostic factor and additional *del 8* or *del 9* E-cadherin further decreases the patient's survival chances.

## MATERIALS AND METHODS

### Patient Selection

Patients with total gastrectomy operated with the diagnosis of adenocarcinoma since 1982 to 2001 in the Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran, with available clinical information and follow-up were considered. The clinical variables obtained from the charts were age at diagnosis, gender, survival time and cause of death. A blinded review of all the slides was made by pathologists and a diagnosis according to Laurén's classification of gastric adenocarcinoma was made. Mestizo Mexican patients with available paraffin material and a morphologic diagnosis of poorly differentiated intestinal, mixed or diffuse-type adenocarcinoma in which UICC staging criteria could be reproduced, were included.

### EGFR Immunohistochemistry

A hematoxylin/eosin (H&E) stained section was obtained and reviewed for morphologic confirmation and two consecutive sections were mounted on charged slides for immunohistochemistry. Immunostainings for EGFR were performed using the Dako EGFRpharmDx™ assay detection system (Dako Corporation, Carpinteria, CA) which recognizes a 170 kDa transmembrane receptor encoded

by the human HER1 gene. The manual staining protocol was precisely followed, and no substitutions were made. After dewaxing in fresh xylene, 100 % ethanol, 95 % ethanol and 70 % ethanol (4 baths each), the slides were placed in a humid chamber for proteolytic digestion with proteinase K solution (100 µl for 5 min), and quenching of endogenous peroxidase for 5 min. The primary antibody was incubated for 30 min followed by 30 min incubation with labelled polymer, and DAB localization of the positive cells. Counterstain was made with hematoxylin followed by 10 slide dips in a bath containing 37 mmol/l ammonia water. In every run control slides were included which were provided to validate the performance of the reagents of the Dako EGFRpharmDx™ assay detection kit. The control slides contained sections of pelleted, formalin-fixed, paraffin-embedded cell line HT-29 with a moderate level of EGFR protein expression (positive control, IHC staining score of the cell pellet is  $2.5 \pm 0.5$ ) and of the EGFR negative CAMA-1 cell line (negative control, score 0).

#### **EGFR reactivity evaluation**

Membrane staining was evaluated in the neoplastic cells and quantified and graded as recommended in the detection kit:

- |          |   |
|----------|---|
| 0 score  | No staining observed, or membrane staining in <10% neoplastic cells. Negative.            |
| 1+ score | Weak complete and/or incomplete membrane staining in >10% neoplastic cells. Positive.     |
| 2+ score | Moderate complete and/or incomplete membrane staining in >10% neoplastic cells. Positive. |
| 3+ score | Strong complete and/or incomplete membrane staining in >10% neoplastic cells. Positive.   |

#### **E-cadherin Immunohistochemical Analysis**

Immunohistochemistry was performed on an automated immunostainer (Ventana Medical Systems, Inc., Tucson, AZ) according to the company's protocols, with minor modifications. Formalin-fixed and paraffin-embedded sections from primary tumors were analyzed. After deparaffinization and rehydration, the slides were

placed in a pressure cooker in 0.01 mol/L citrate buffer (pH 6.0) containing 0.1% Tween 20 and heated in a microwave oven at maximum power for 30 min. The sections were cooled in Tris-buffered saline and washed in 3% goat serum for 20 min. The antibodies used included anti-E-cadherin antibody AEC (clone 36, Transduction laboratories, Lexington, NY, dilution 1:1000) and mutation specific *del 8* and *del 9* E-cadherin antibodies that were produced in our laboratory and reported elsewhere (Becker et al, 1999; 2002). Appropriate positive controls were used to confirm the adequacy of the staining. AEC reactivity was defined as normal (membranous), atypic (partial membran staining, cytoplasmic or heterogenous staining) or negative staining. *del 8* and *del 9* E-cadherin was considered positive when membranous staining was observed.

### Statistical analysis

Statistical analyses were performed using Fisher's exact, K and  $\chi^2$  tests when appropriate. Kaplan-Meier survival time analysis was used to correlate EGFR reactivity, localization of positive cells (surface or deep), pT, pN and pM status with clinical evolution. Cox regression analysis was performed correlating EGFR reactivity, localization of positive cells, and stage with prognosis. A two sided p value less than 0.05 was considered to be statistically significant.

## RESULTS

### CLINICOPATHOLOGICAL FEATURES

The clinicopathological features of 92 Mestizo Mexican gastric cancer patients are shown in Table 5. Median patient age at the time of diagnosis was 58 years (range of 14-86 years). 47 patients (51 %) were female, 45 patients (49 %) were male. Classification of gastric cancer histotype according to Laurén was poorly differentiated intestinal type in 37 cases (40 %), diffuse type in 51 tumor samples (55 %), mixed type with intestinal and diffuse components in 4 cases (5 %). The stages according to the UICC classification were IA, B in 3 patients (3 %), II in 25 patients (27 %), IIIA in 20 patients (22 %), IIIB in 14 cases (15 %) and IV in 30 cases (33 %).

**EGFR Score of Reactivity and *del 8* or *del 9* E-cadherin reactivity**

Gastric carcinomas were stained with standardized EGFR immunohistochemical detection systems. Membrane staining was evaluated in the neoplastic cells and quantified and graded as recommended in the detection kits (Table 6). For EGFR expression, 49 cases (53.0 %) were negative or reactive in <10 % of neoplastic cells (score 0). Complete and/or incomplete membrane staining in >10 % of neoplastic cells was weak in 17 tumors (18.5 %, score 1+), moderate in 17 adenocarcinomas (18.5 %, score 2+) and strong in 9 neoplasms (10.0 %, score 3+). Nerve and muscle cells were reactive and served as reactive internal control. Normal gastric mucosa was negative for EGFR expression. EGFR reactivity showed a broad variability with a range from few positive tumor cells surrounded from a majority of negative neoplastic cells to equal EGFR expression in almost all tumor cells.

*del 8* or *del 9* E-cadherin expression was investigated using mutation-specific anti-E-cadherin antibodies (Becker et al, 1999; 2002, loc. cit.). *del 8* or *del 9* E-cadherin staining was observed in 10/92 cases (10.9 %).

**Influence of EGFR Score, *del 8* or *del 9* E-cadherin Reactivity, and Stage on Survival**

In an univariate analysis, the correlation of EGFR reactivity score, *del 8* or *del 9* E-cadherin reactivity, and stage with prognosis was investigated (Table 7). A statistically significant association was found between the length of survival and EGFR reactivity score 2+/3+ ( $p=0.002$ ) as well as stage III/IV ( $p=0.02$ ).

A multivariate analysis using Cox's proportional hazard model revealed that EGFR score 2+/3+ ( $p=0.012$ ) and stage III/IV ( $p=0.028$ ) were significantly correlated with a decrease in survival. The presence of *del 8* or *del 9* E-cadherin reactivity is disadvantageous for the patients. The relative risk to die in patients with EGFR score 2+/3+ and *del 8* or *del 9* E-cadherin reactivity, was elevated 2.454 fold x 2.142 fold (5.256 fold). Consequently, *del 8* or *del 9* E-cadherin reactivity is an additional risk factor.

### **Influence of EGFR Score, *del 8* or *del 9* E-cadherin Reactivity and Stage on Survival in diffuse and mixed type Gastric Carcinomas**

Previous observations have demonstrated the presence of E-cadherin mutations in diffuse and mixed type, but not in intestinal type gastric carcinomas (Becker et al, 1994, loc. cit.). In the present study with tumors from Mexican patients, *del 8* or *del 9* E-cadherin reactivity was found in 6 diffuse, 3 mixed and 1 intestinal type gastric carcinomas (Gamboa-Dominguez et al, in preparation).

EGFR reactivity score, *del 8* or *del 9* E-cadherin reactivity, and stage were correlated with prognosis in diffuse and mixed type cases (Table 8, univariate all patients). Only stage III/IV was statistically correlated with patient survival ( $p=0.007$ ). A multivariate analysis using Cox's proportional hazard model revealed that *del 8* or *del 9* E-cadherin reactivity was more important for prognosis in diffuse and mixed type gastric carcinomas than EGFR score 2+/3+, this trend was observable ( $p=0.174$ ). *del 8* or *del 9* E-cadherin reactivity is an additional risk factor. Stage III/IV ( $p=0.005$ ) was significantly correlated with a decrease in survival.

### **Influence of *del 8* or *del 9* E-cadherin reactive neoplastic cells or EGFR score and stage on Survival**

Kaplan Meier method was used to correlate stage and *del 8* or *del 9* E-cadherin reactivity with patient survival (Fig. 40). In the presence of *del 8* or *del 9* E-cadherin reactivity, survival time of patients in stage I/II or III/IV was significantly decreased. The log rank test indicates a global  $p$  value 0.009.

Kaplan Meier method was used to investigate the correlation between EGFR expression and stage with patient survival (Fig. 41). In the presence EGFR reactivity, survival time of patients in stage III/IV was decreased. The log rank test indicates a global  $p$  value 0.0326. Taken together, for patients in stage I/II or II/IV, survival prognosis is poorer in the presence of EGFR or *del 8* or *del 9* E-cadherin reactivity. Taken together, the data suggest that expression of EGFR as well as presence of *del 8* or *del 9* E-cadherin reactivity in combination with tumor stage contribute to poor prognosis. Therefore, treatment of patients with these two abnormalities is highly recommended.



**Tables relating to the examples:**

Table 1: Clinicopathologic features of 89 patients with gastric cancer.

Age	years		
Mean	57.8		
Median	60.0		
Standard deviation	15.2		
Range	14 – 86		
	n	%	
Gender			
Female	44	49.4	
Male	45	50.6	
Histotype (Laurén)			
Intestinal	36	40.4	
Diffuse	49	55.1	
Mixed	4	4.5	
Stage (UICC)			
IB	1	1.1	
II	25	28.1	
IIIA	20	22.5	
IIIB	14	15.7	
IV	29	32.6	
Residual Disease			
R0	69	77.5	
R1	20	22.5	

**Table 2: EGFR reactivity in 89 patients with gastric cancer.**

## EGFR score of reactivity

	n	%
0	47	52.8
1+	17	19.1
2+	16	18.0
3+	9	10.1

## Percentage of EGFR positive cells

	n	%
0 %	26	29.2
<10 %	21	23.6
10-50 %	30	33.7
>50 %	12	13.5

**Table 3. EGFR score of reactivity and its correlation with clinicopathological features and morphology in 89 Mexican Mestizo patients with gastric cancer.**

		EGFR score				
		0	1+	2+	3+	total
Histotype (Laurén)						
	Intestinal	23	4	7	2	36
	Mixed	1	2	0	1	4
	Diffuse	23	11	9	6	49
p=0.201						
Tumor invasion						
	pT 2	7	1	0	0	8

93					
pT 3-4	40	16	16	9	81
p=0.304					
Perigastric lymph node status					
pN0	13	5	1	2	21
pN1-2	34	12	15	7	68
p=0.313					
Distant metastases					
pM0	40	15	7	9	71
pM1	7	2	9	0	18
p=0.002					
Residual disease					
R0	39	13	10	7	69
R1	8	4	6	2	20
p=0.406					
Clinical stage (UICC)					
I-II	19	5	1	1	26
III-IV	28	12	15	8	63
p=0.033					

**Table 4: Analysis of prognostic factors in gastric carcinomas**

**univariate**

	Significance (p value)
EGFR reactivity score	0.003
Percentage of EGFR reactive neoplastic cells	0.071
EGFR reactive cells in mucosa	0.019
EGFR reactive cells in submucosa	0.124
EGFR reactive cells in muscularis or subserosa	0.001
EGFR reactive cells in submucosa, muscularis or subserosa	0.002

Stage III-IV	0.064
Distant metastases	0.0001
Residual Disease	0.012

**multivariate: Cox proportional hazard model in a stepwise forward fashion**

	Significance (p value)	95% CI for relative risk		
		relative risk	Lower	Upper
EGFR reactive cells in muscularis or subserosa	0.004	2.679	1.373	5.224
Distant metastases	0.016	2.583	1.190	5.607
Residual Disease	0.039	2.057	1.037	4.082

CI: confidence intervall

**Table 5. Clinicopathologic features of 92 patients with gastric cancer**

<b>Age (median), years</b>	<b>58</b>	<b>(Range 14 – 86)</b>
	n	%
Gender		
Female	47	51
Male	45	49
Histotype (Laurén)		
Intestinal	37	40
Diffuse	51	55
Mixed	4	5
Stage (UICC)		
IA, B	3	3

95

II	25	27
IIIA	20	22
IIIB	14	15
IV	30	33

**Table 6. EGFR score of reactivity in 92 patients with gastric adenocarcinoma**

EGFR Score	n	%
0	49	53.0
1+	17	18.5
2+	17	18.5
3+	9	10.0

**Table 7: Analysis of prognostic factors in gastric carcinomas****univariate**

	Significance (p value)
EGFR reactivity score 2+/3+	0.002
<i>del 8</i> or <i>del 9</i> E-cadherin reactivity	0.853
Stage III/IV	0.020

multivariate: Cox proportional hazard model in a stepwise forward fashion

	Significance (p value)	Relative Risk	95% CI for Relative Risk	
			Lower	Upper
EGFR reactive score 2+/3+	0.012	2.454	1.220	4.934

*del 8-*, *del 9* E-cadherin reactivity

0.125                      2.142                      0.809                      5.671

Stage III/IV

0.028                      2.241                      1.096                      5.002

CI: confidence intervall

Table 8: Analysis of prognostic factors in diffuse and mixed type gastric carcinomas

**univariate**

Significance (p value)

EGFR reactivity score 2+/3+

0.106

*del 8* or *del 9* E-cadherin reactivity

0.690

Stage III/IV

0.007

**multivariate: Cox proportional hazard model in a stepwise forward fashion**

95% CI for Relative Risk

Significance  
(p value)

Relative  
Risk

Lower

Upper

*del 8-*, *del 9* E-cadherin reactivity

0.174

1.948

0.745

5.094

Stage III-IV

0.005

3.687

1.492

9.114

CI: confidence intervall

### Claims

1. Use of (an) EGF receptor antagonist(s)/inhibitor(s) for the preparation of a pharmaceutical composition for the prevention, amelioration or treatment of gastric carcinomas.
2. The use of claim 1 wherein said gastric carcinoma is a diffuse gastric carcinoma.
3. The use of claim 1 or 2 for inhibiting the motility of tumor cells in a subject suffering from said carcinomas.
4. The use of any one of claims 1 to 3 wherein the carcinoma cells of the patients suffering from said carcinomas do not comprise an overexpression of EGF receptor.
5. The use of any one of claims 1 to 4 wherein the cells derived from said carcinomas comprise at least one mutation in the  $\beta$ -catenin signal transduction pathway.
6. The use of any one of claims 1 to 4 wherein cells derived from said carcinoma comprise a mutation in E-cadherin.
7. The use of claim 6 wherein said E-cadherin mutation is selected from the group consisting of a full or partial deletion of exon 8, a full or partial deletion of exon 9, a full or partial deletion of exon 10 and one or more point mutations.
8. The use of any one of claims 1 to 7, whereby said EGF receptor antagonist(s)/inhibitor(s) inhibits/inhibit the motility or metastasis formation

of cells comprising at least one mutation in the  $\beta$ -catenin signal transduction pathway.

9. A method for the treatment of gastric carcinomas as defined in any one of the claims 1 to 8 comprising the administration of (an) EGF-receptor antagonist(s)/inhibitor(s) to a subject in need of such a treatment.
10. The use of any one of claims 1 to 8 or the method of claim 9 whereby the EGF-receptor antagonist/inhibitor is selected from the group consisting of an anti-EGF-receptor antibody or a derivative or a fragment thereof, an EGF-toxin or immunotoxin, antisense oligonucleotides specifically interacting with nucleic acid molecules encoding EGFR, siRNA or RNAi directed against EGFR, ribozymes specifically interacting with EGFR nucleic acid molecules or tyrosine kinase inhibitors.
11. The use or the method of claim 10, wherein said EGF-toxin is conjugated to Pseudomonas exotoxin A or a truncated version thereof whereby said EGF-toxin is fused to genistein.
12. The use or the method of claim 10, wherein said tyrosine kinase inhibitor is tyrphostin AG1478, ZD-1839, OSI-774, PKI-166, PD 158780, CPG 59326 or CI-1033.



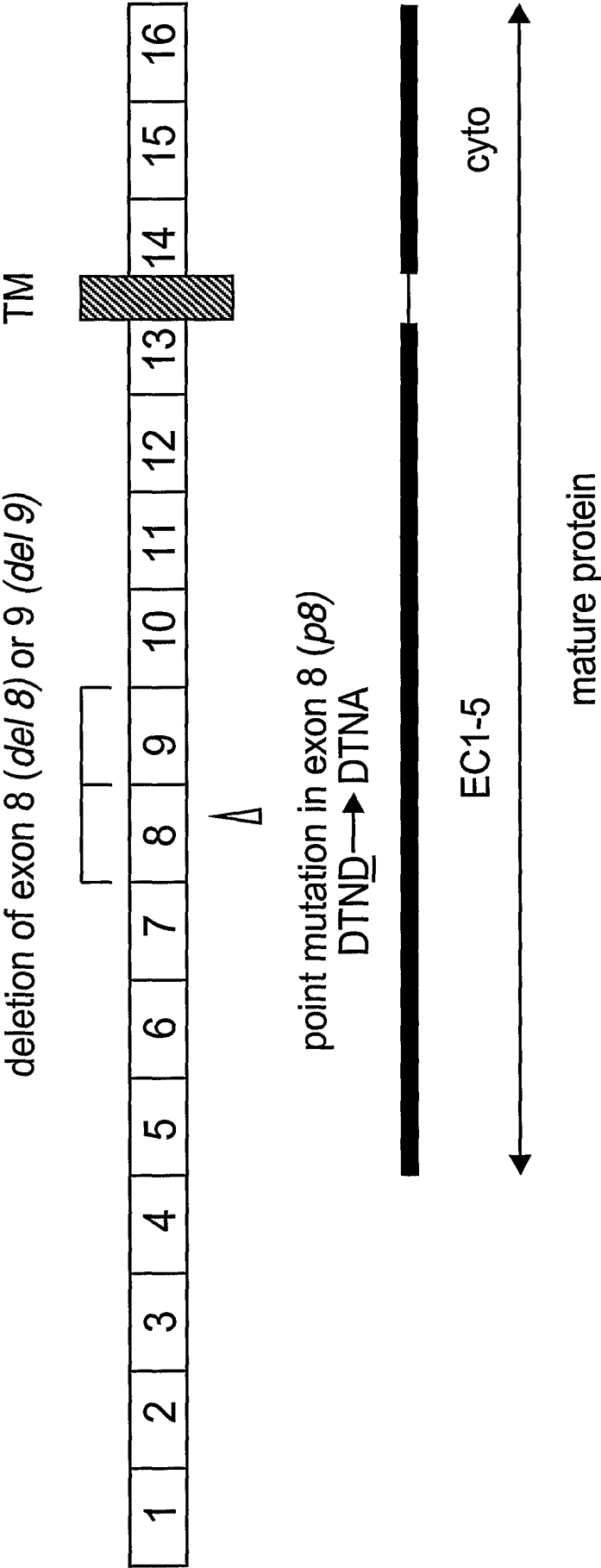


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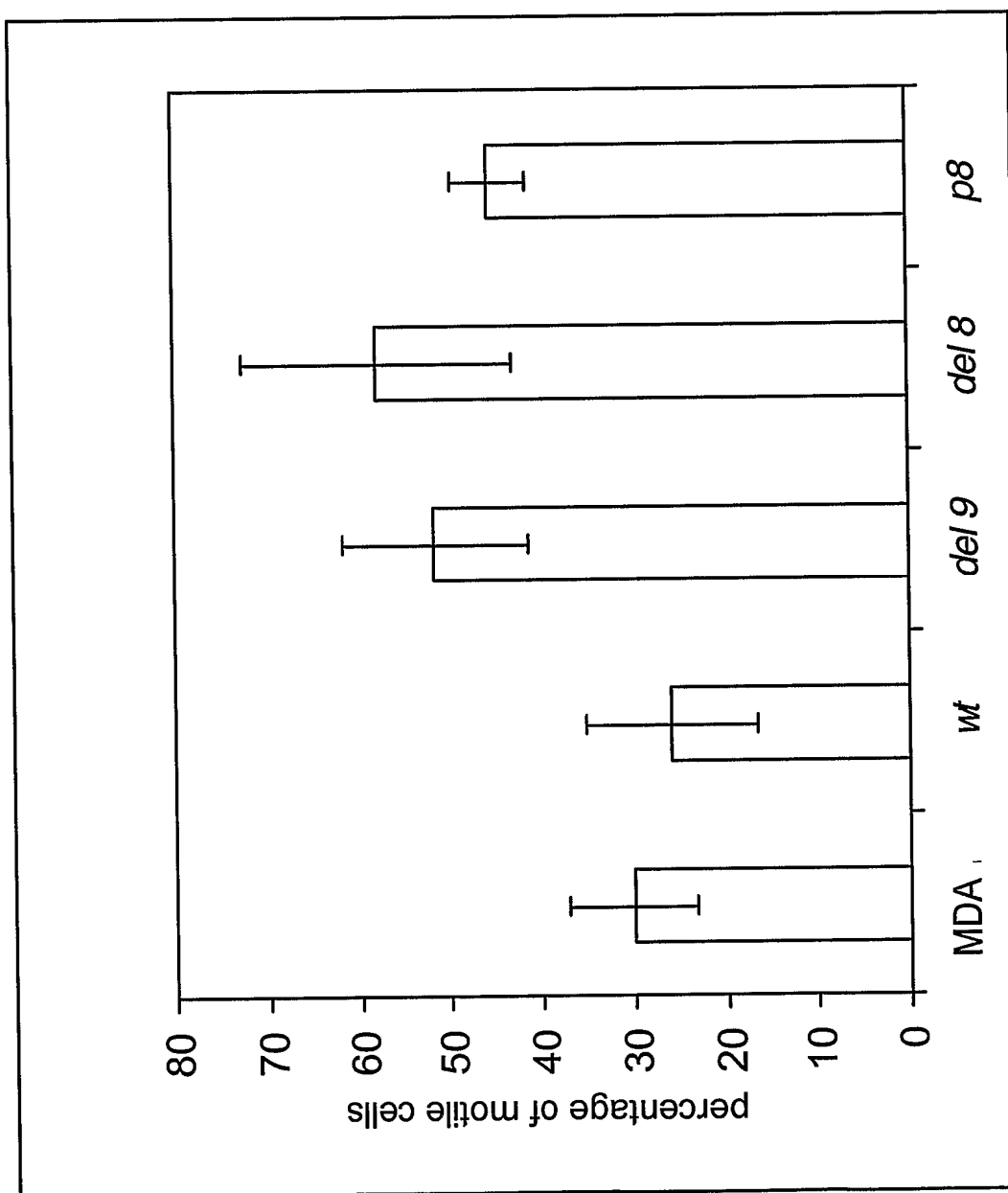


Figure 1 B

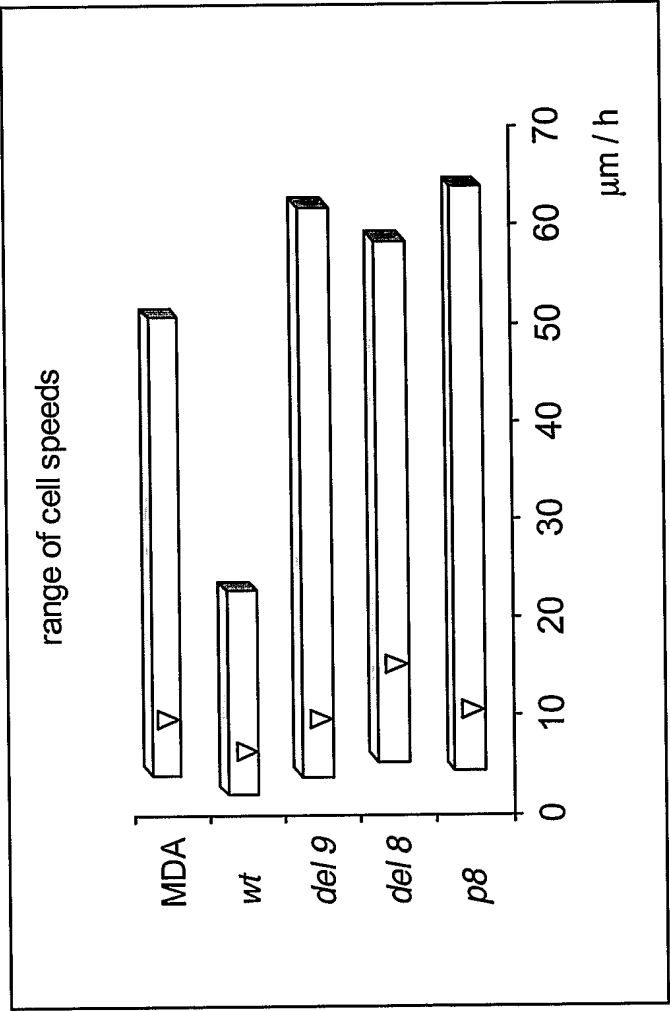
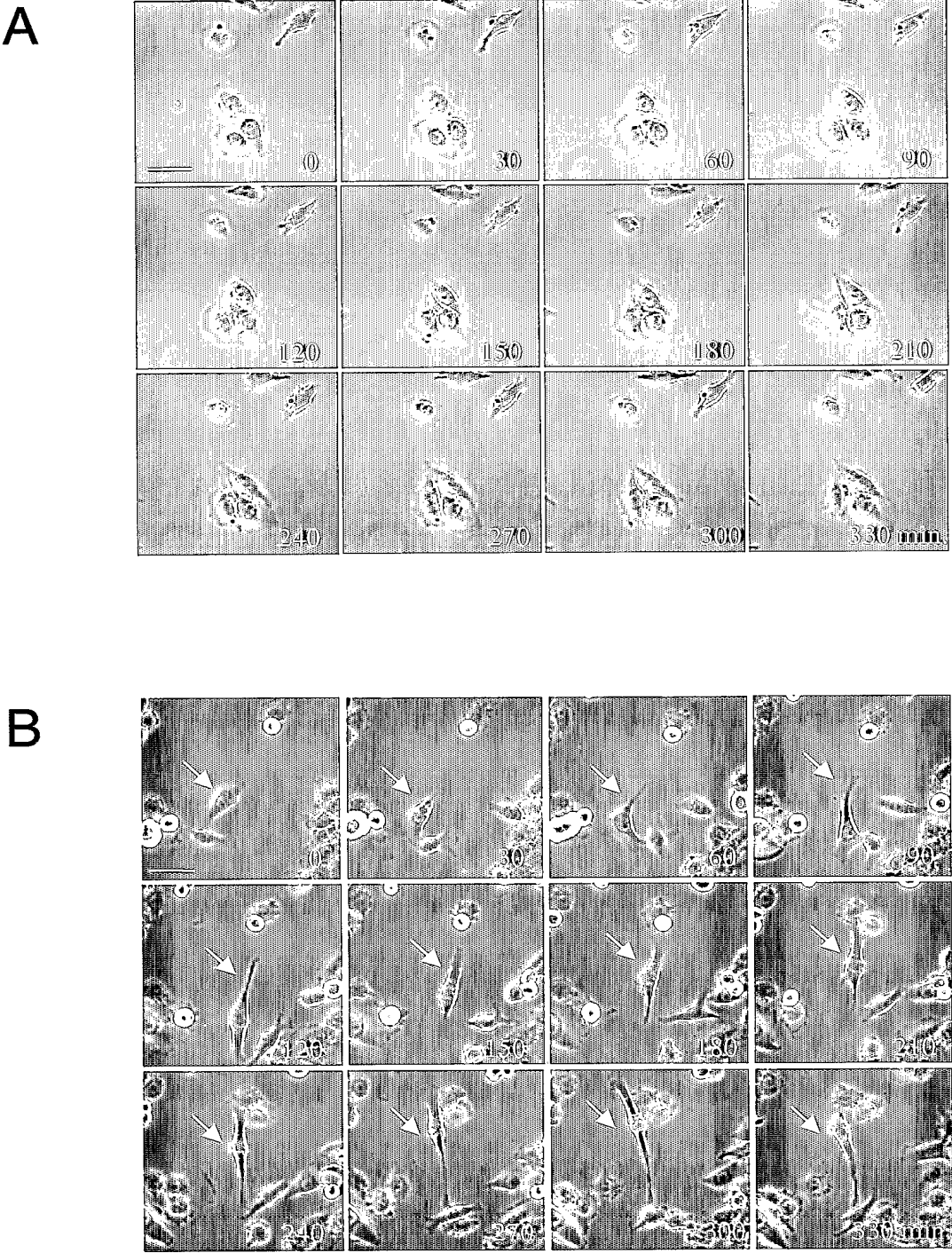
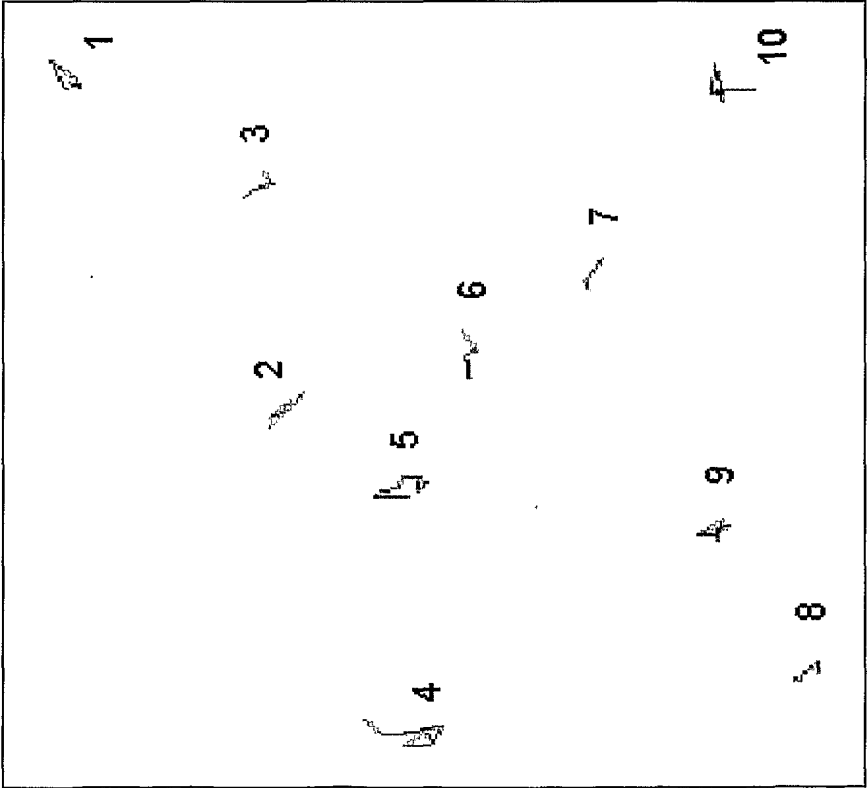


Figure 1 C

Figure 2



*wt*-E-cadherin



*del* 8-E-cadherin

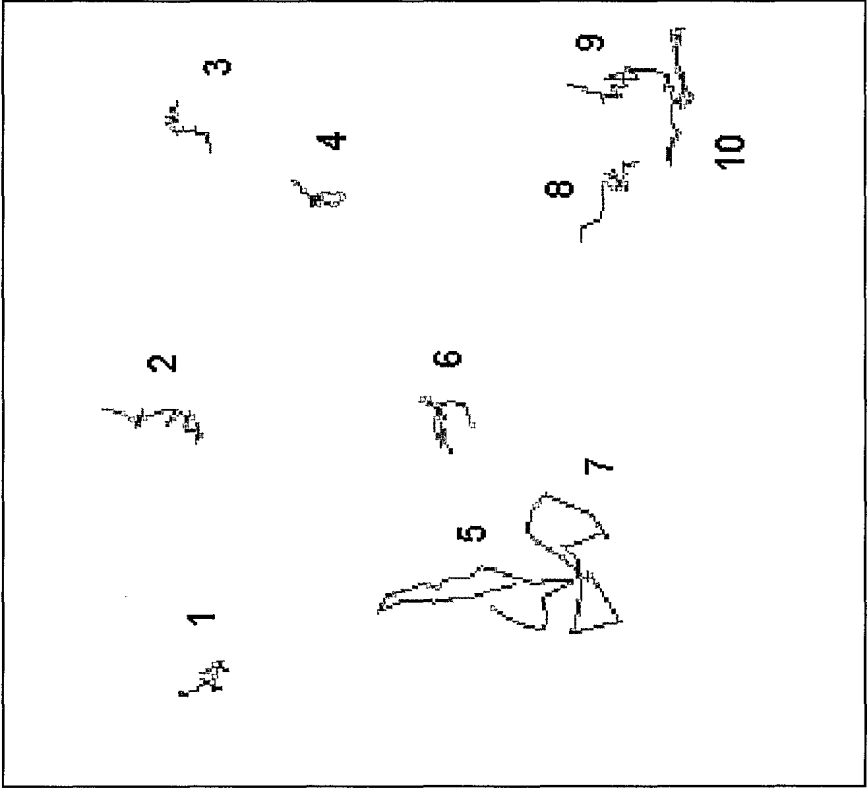


Figure 3

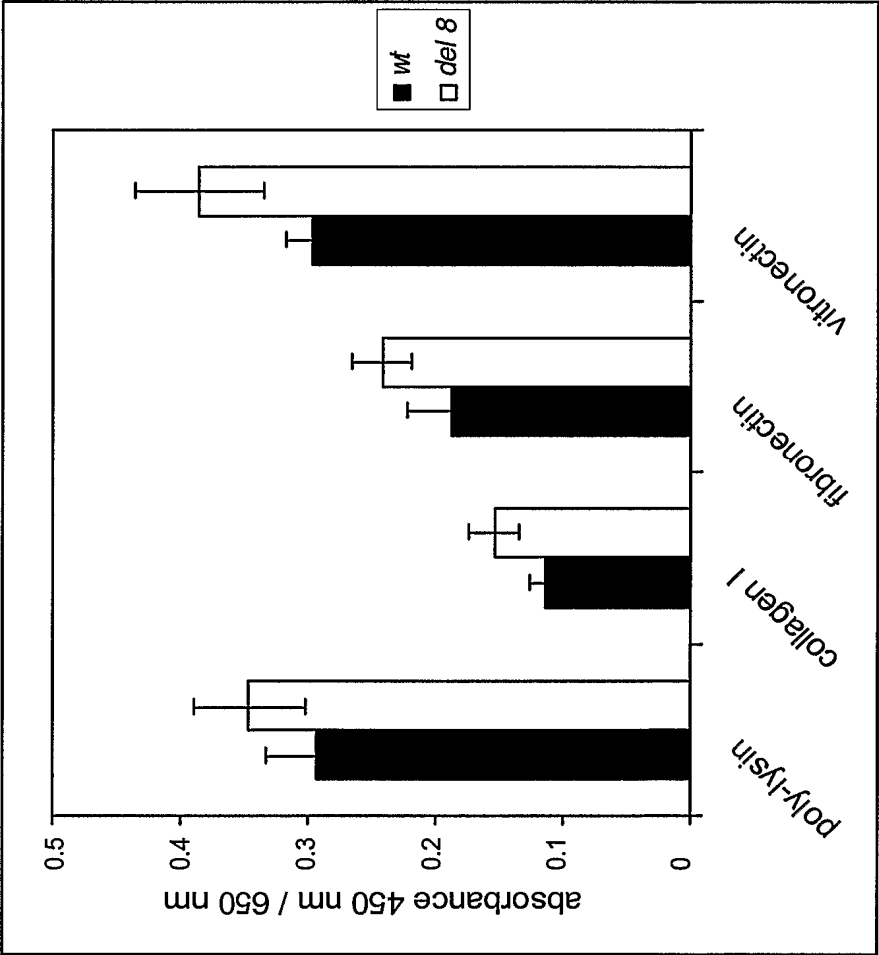


Figure 4 A

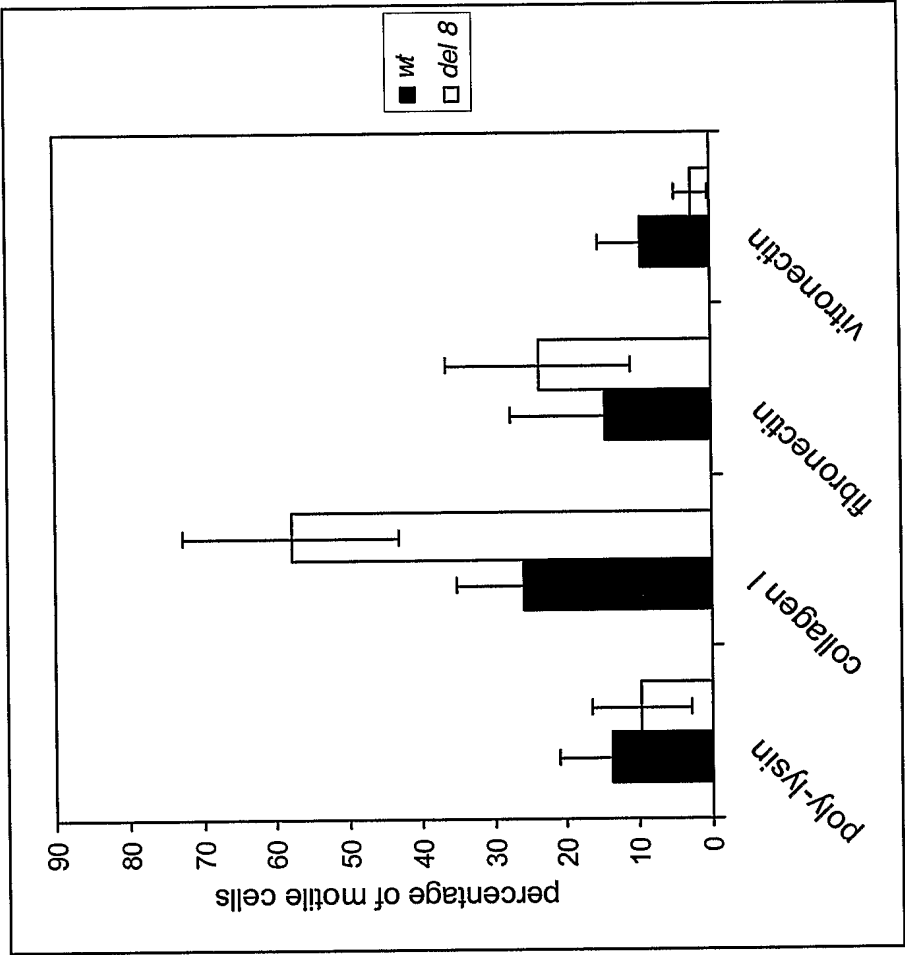


Figure 4 B

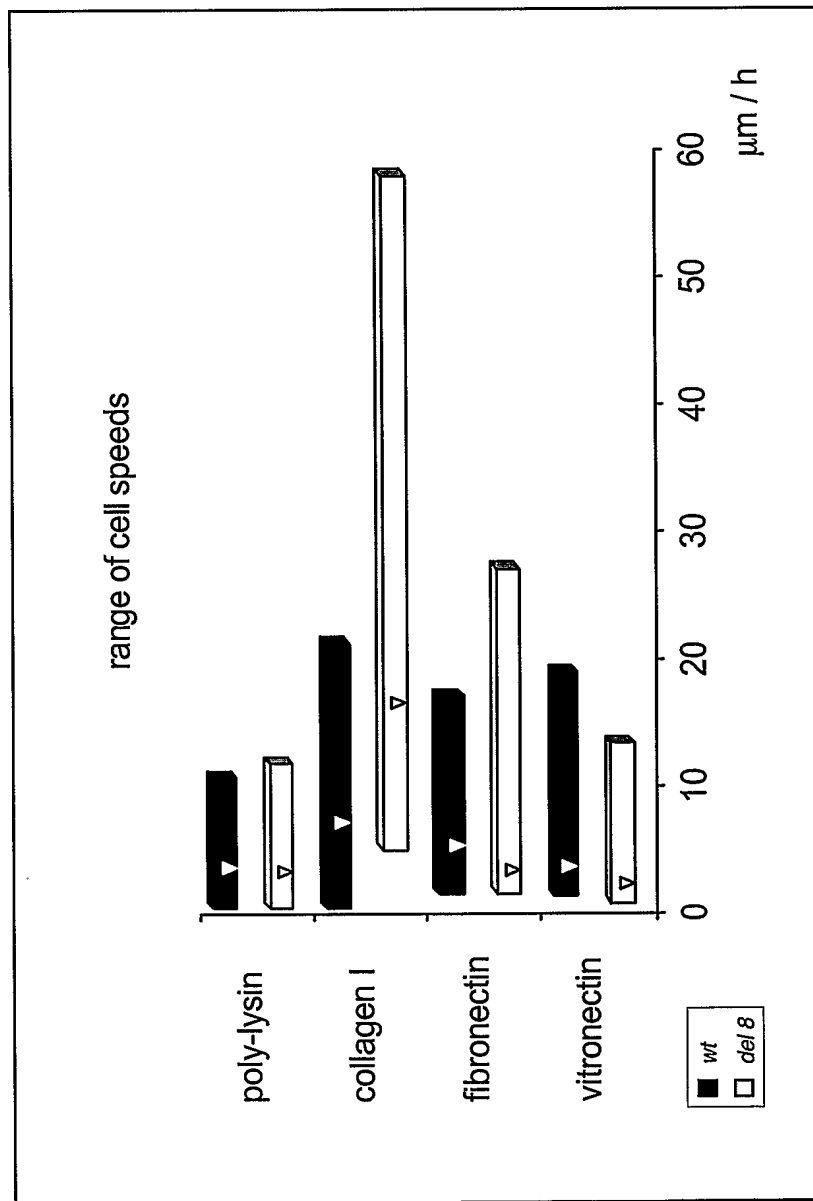
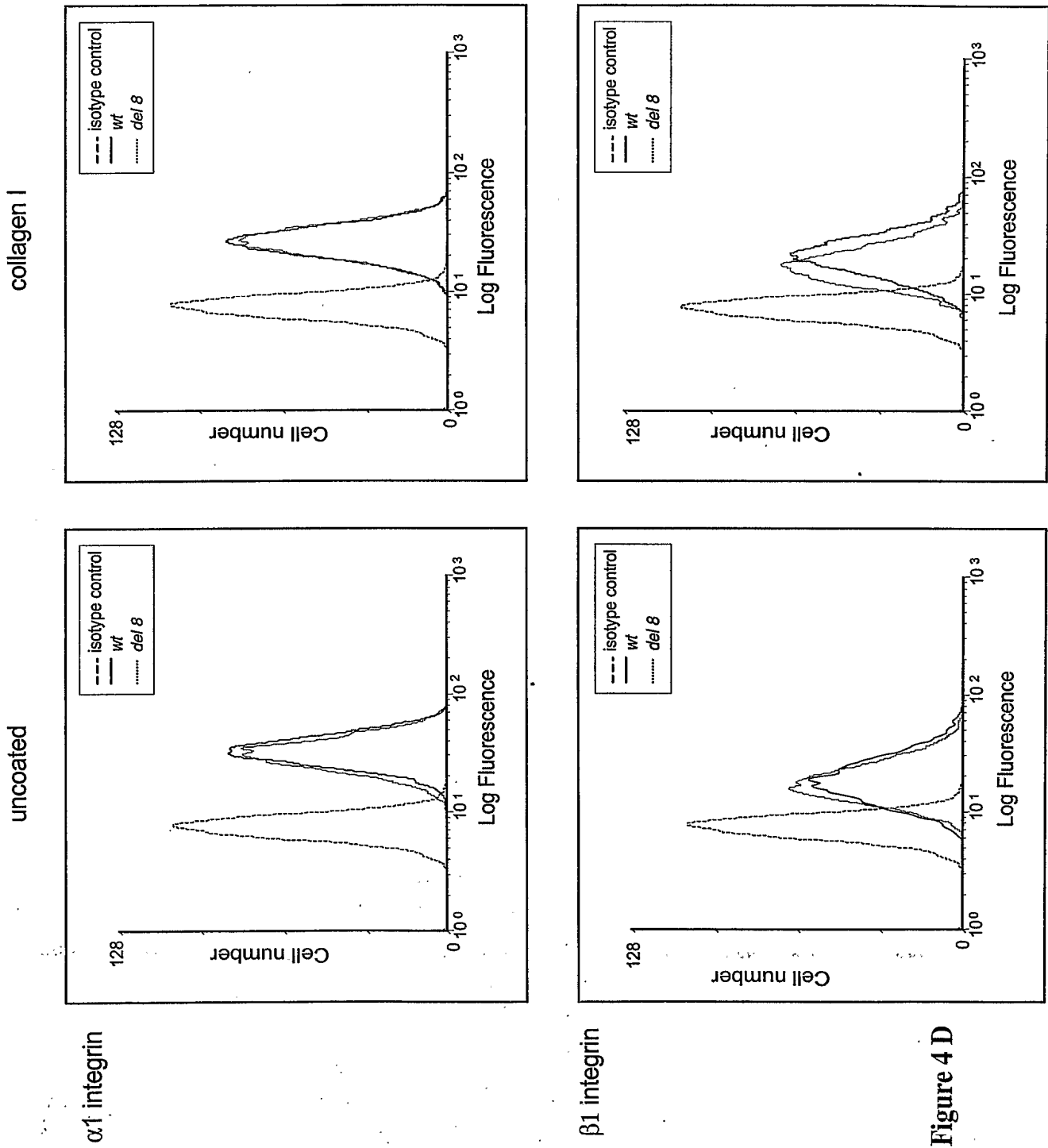


Figure 4 C





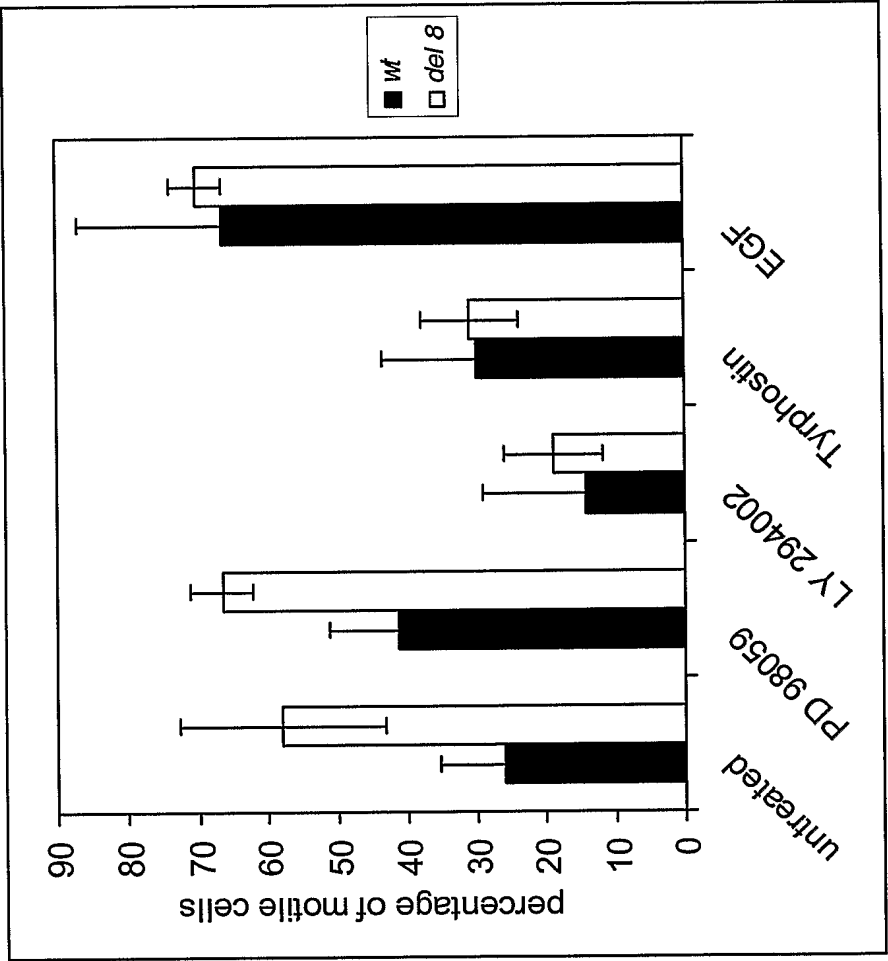


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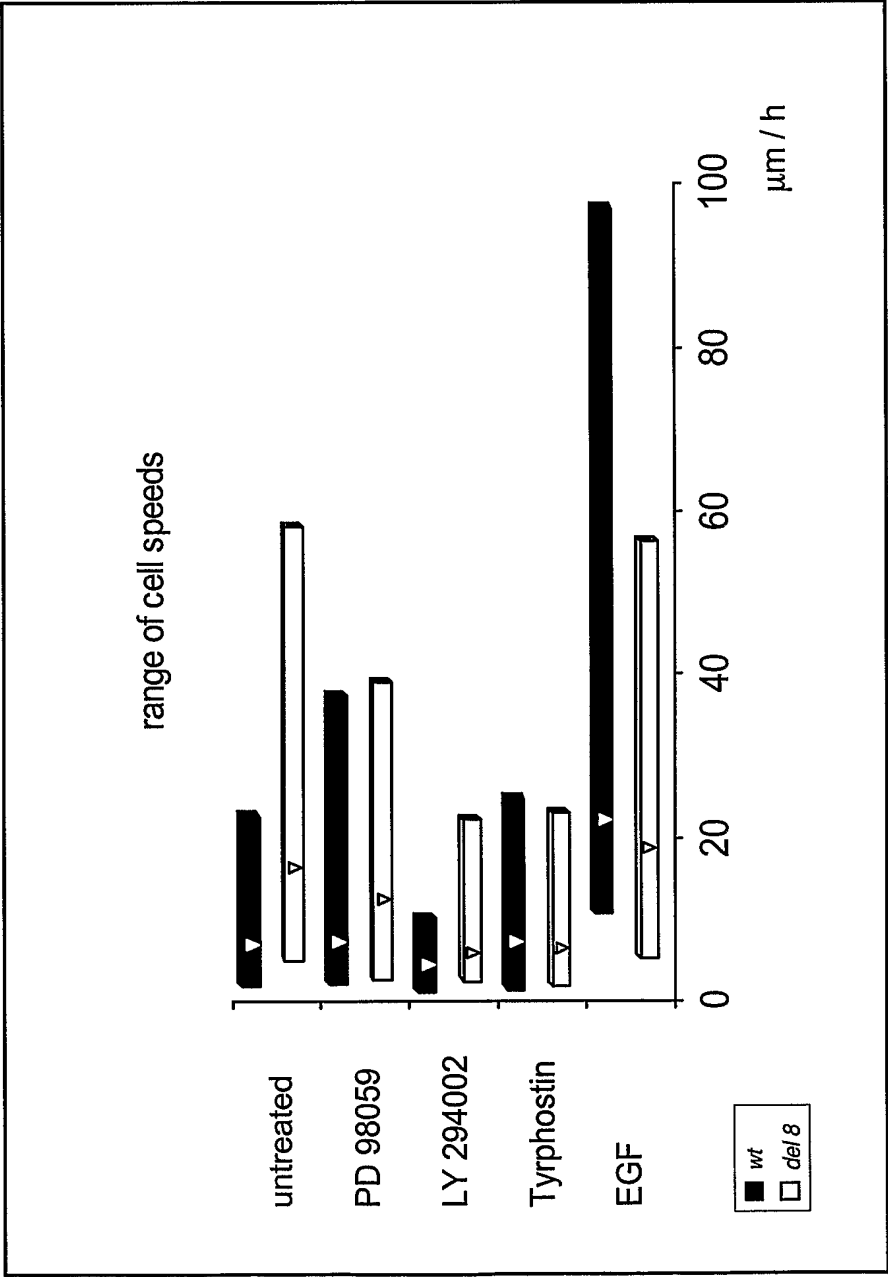


Figure 5 B

Figure 5

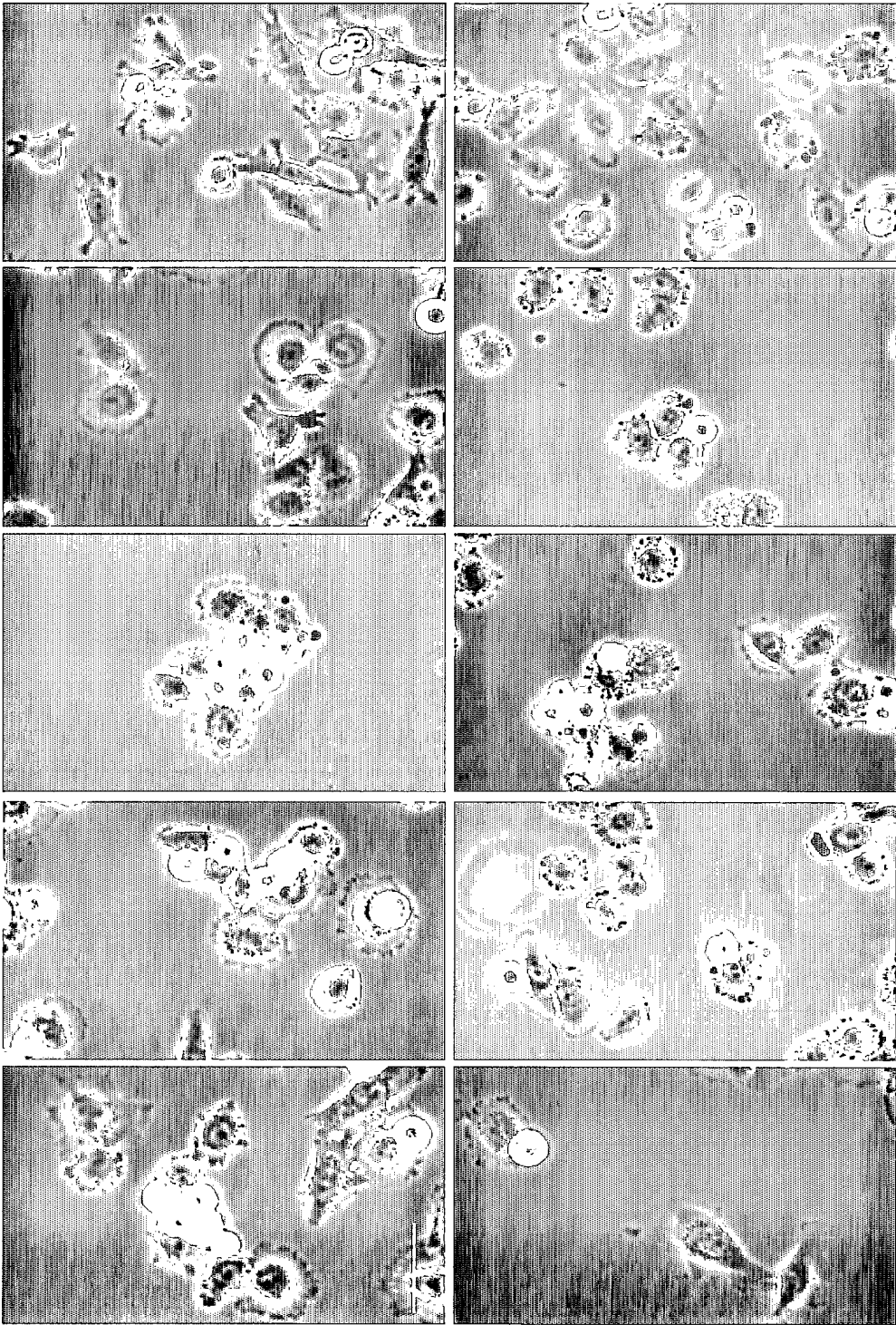
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Tyrphostin

LY 294002

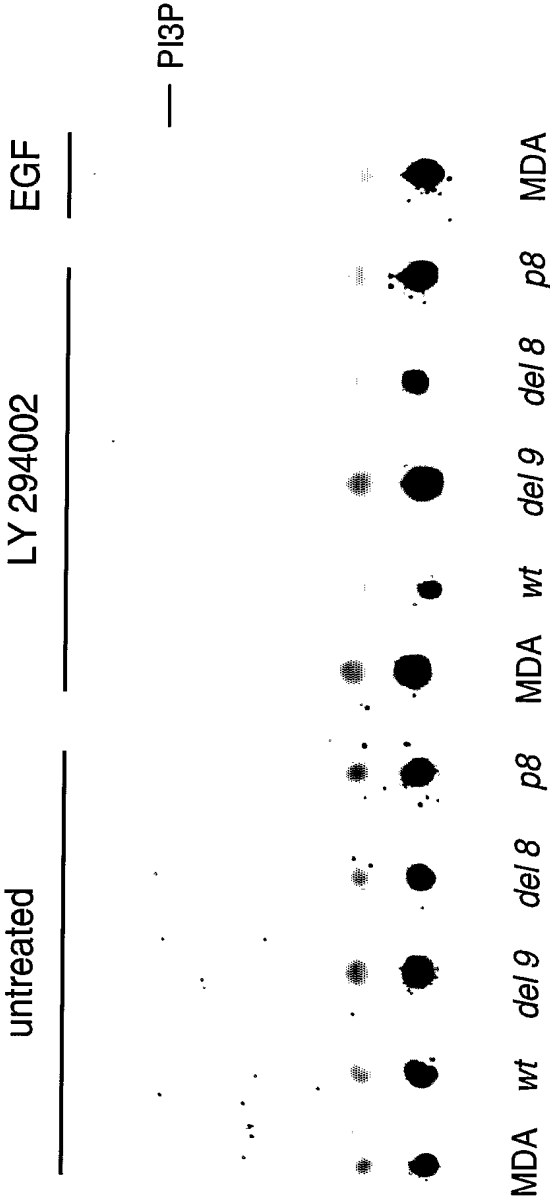
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untreated



C

Figure 6



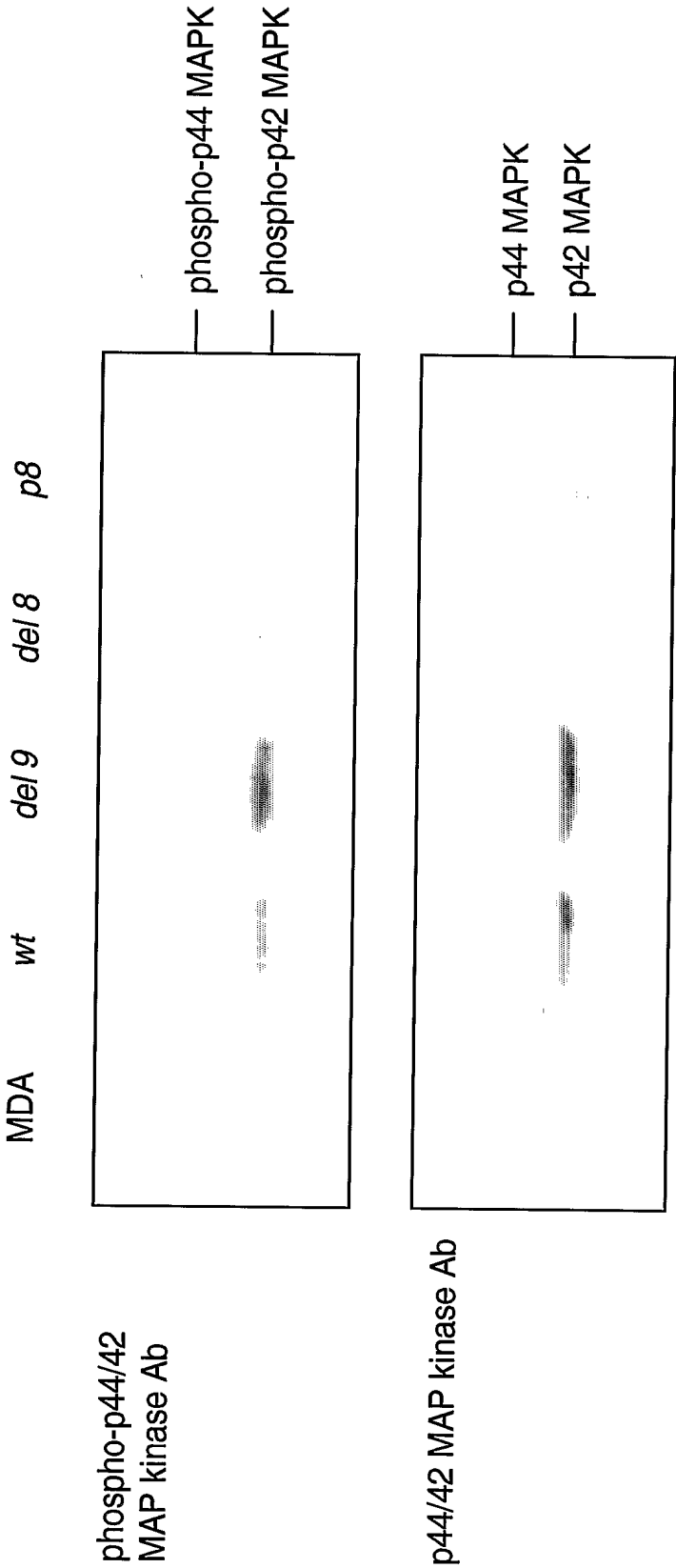


Figure 7 A

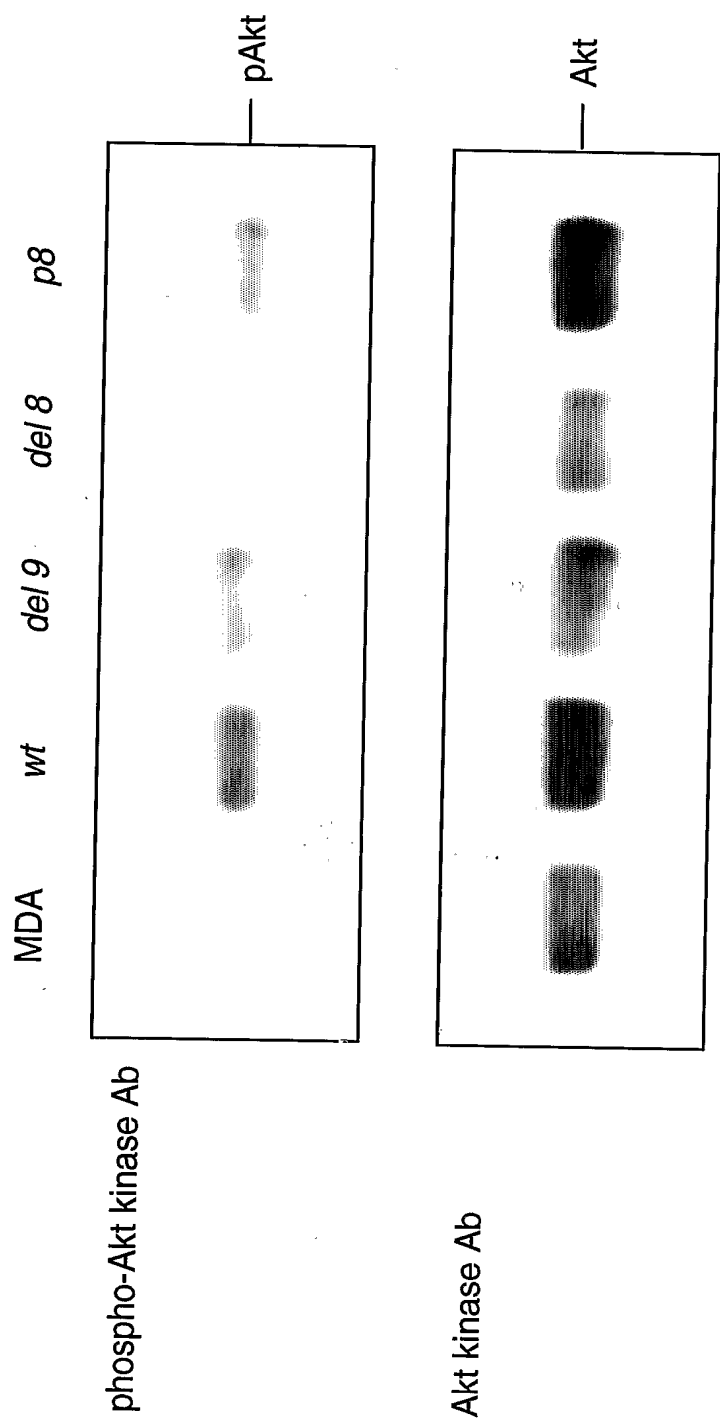


Figure 7 B

Figure 8

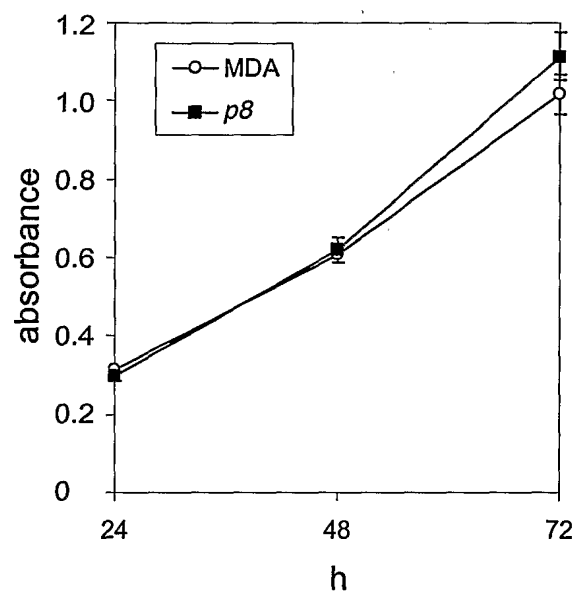
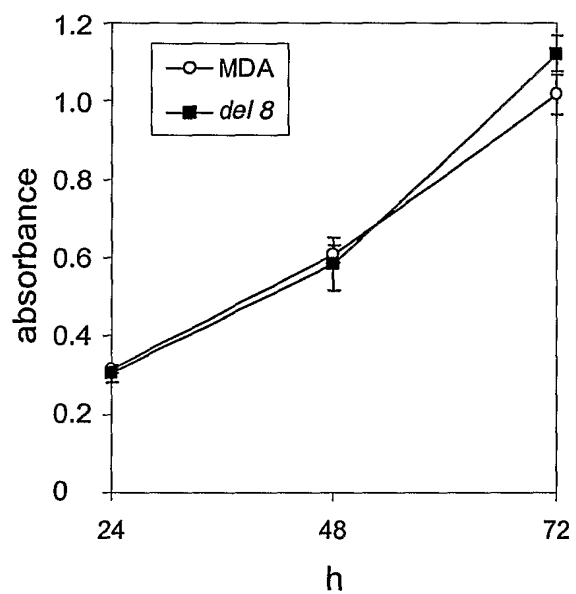
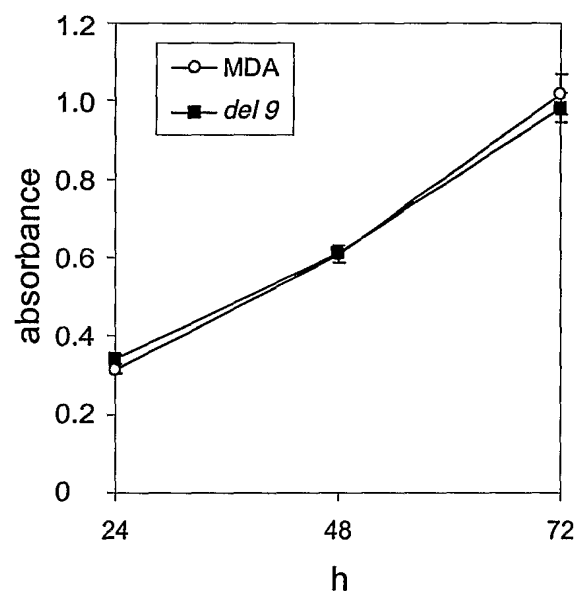
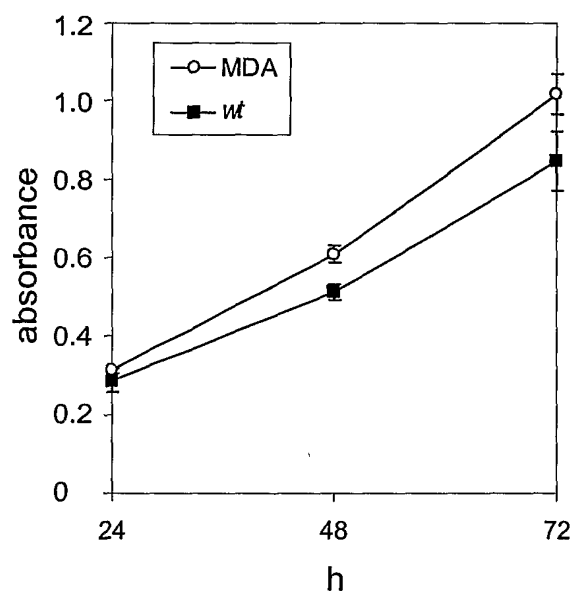




Figure 9

A

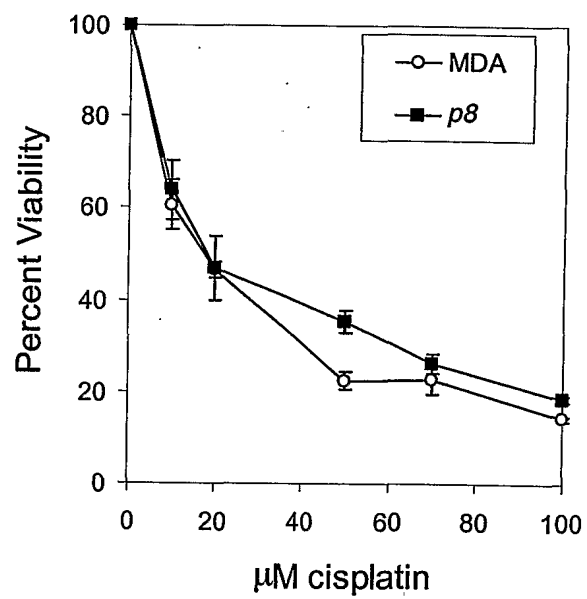
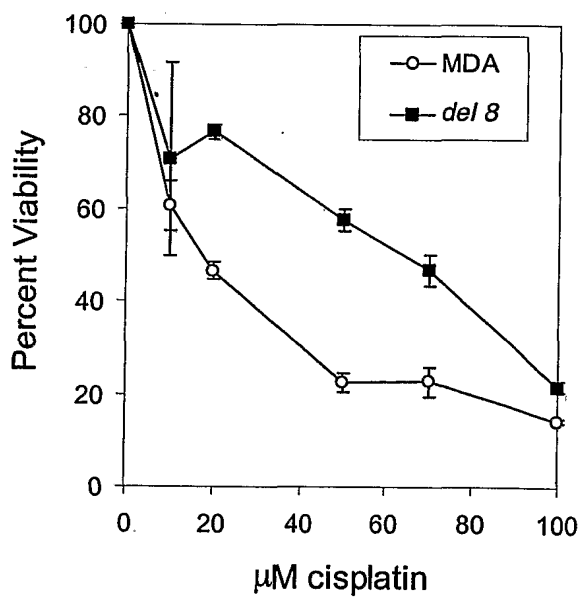
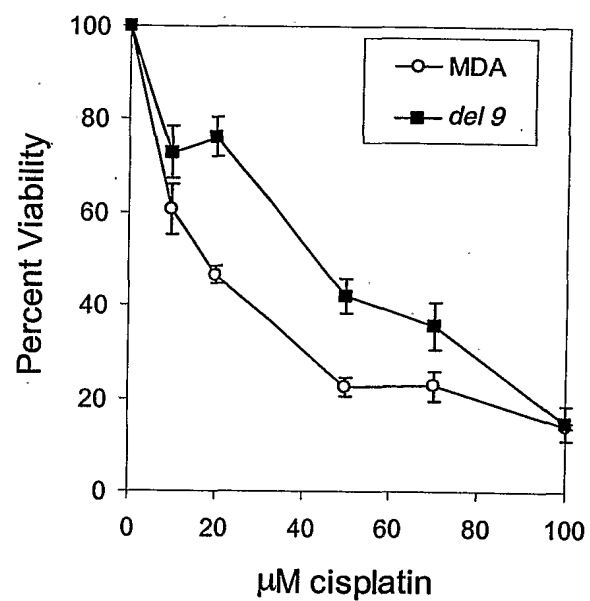
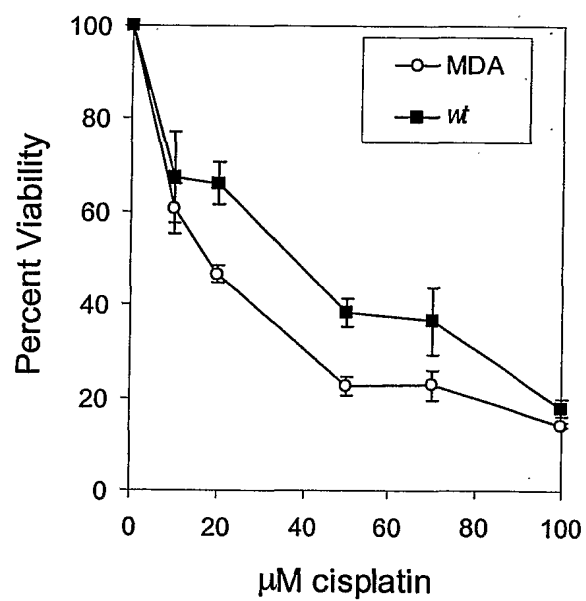


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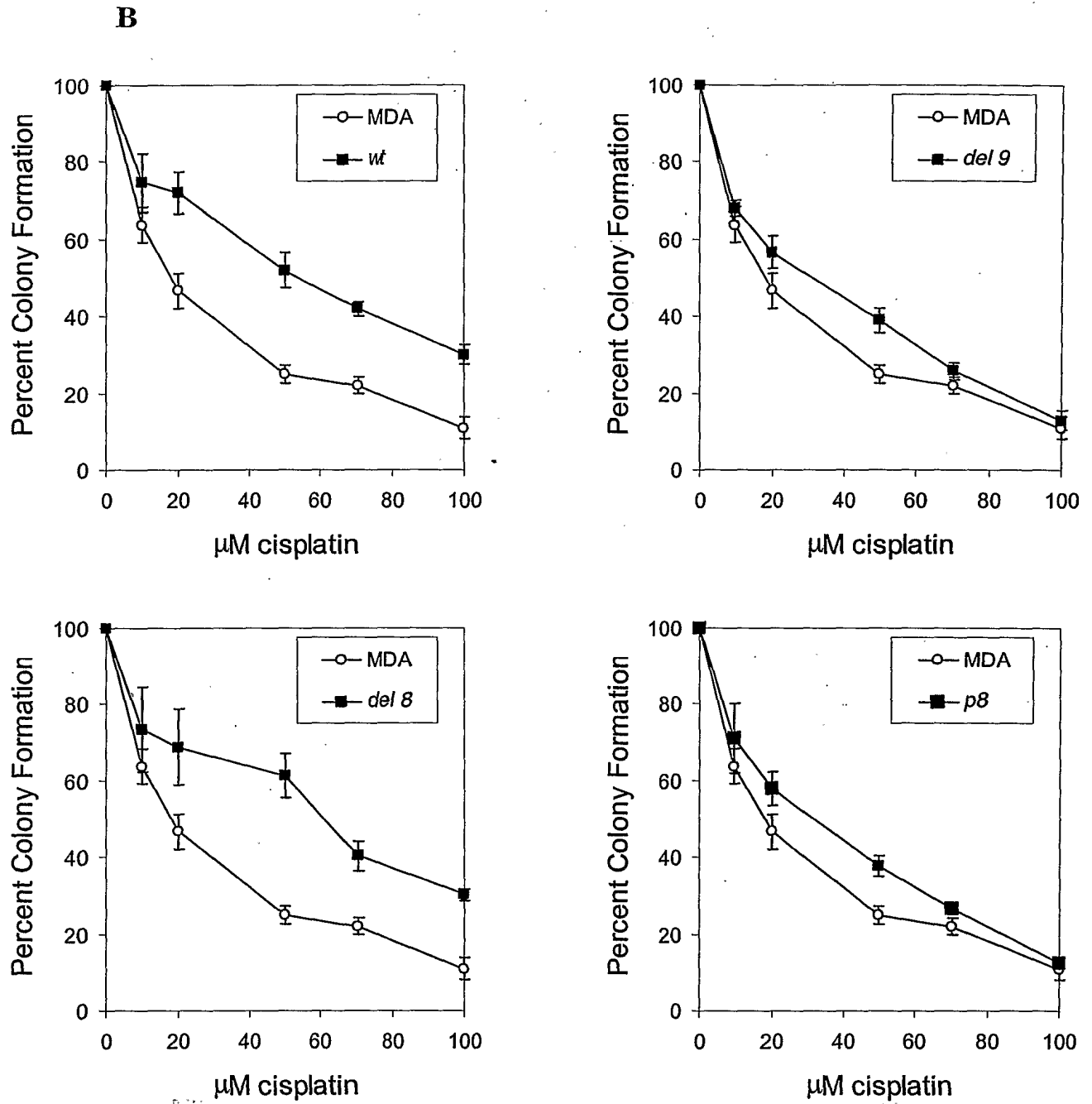


Figure 10

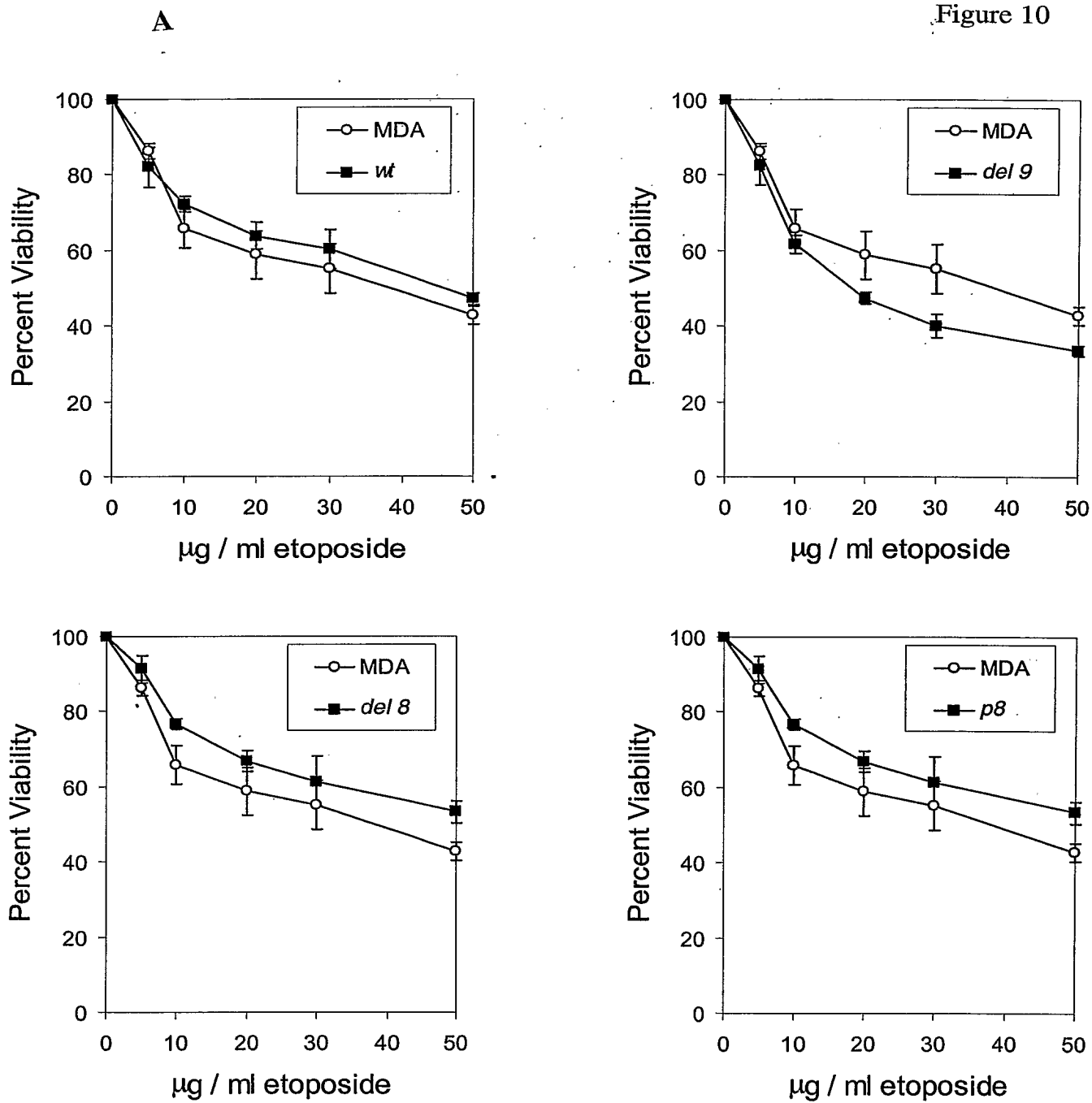


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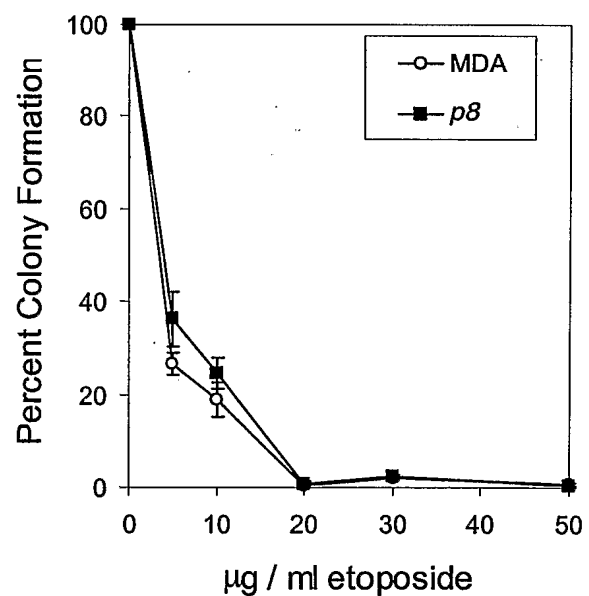
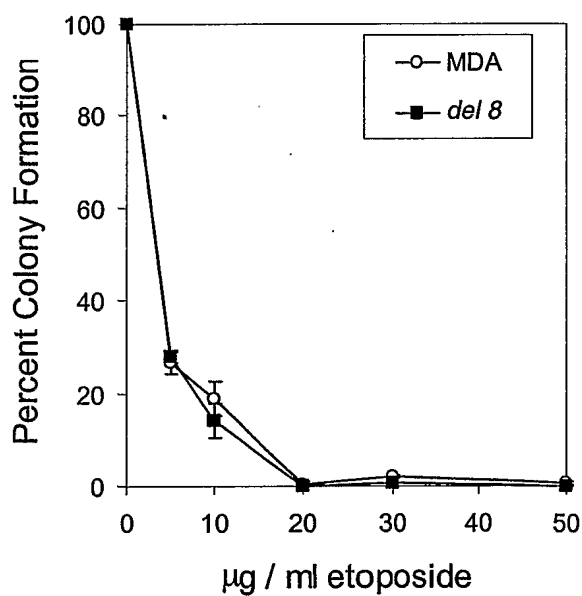
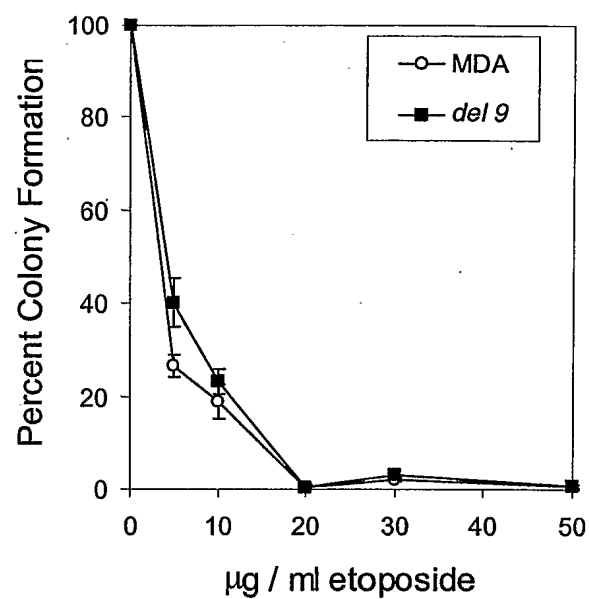
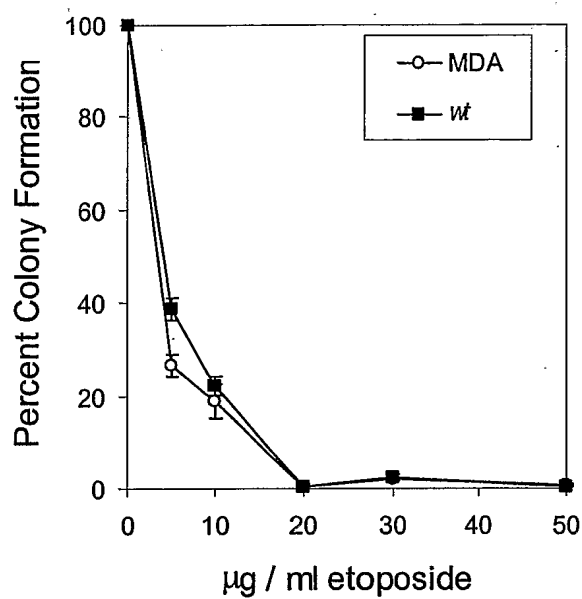
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Figure 11

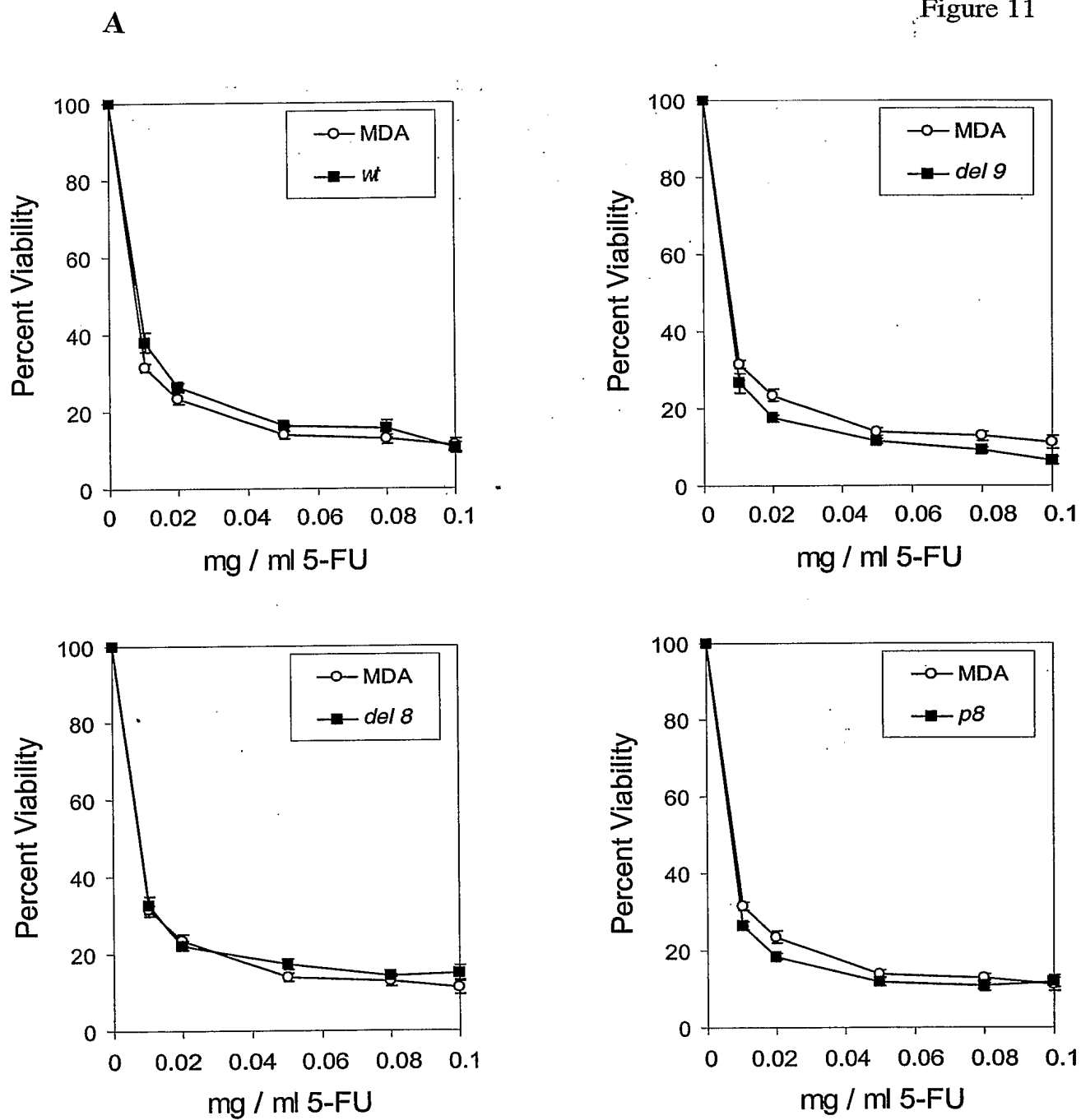


Figure 11

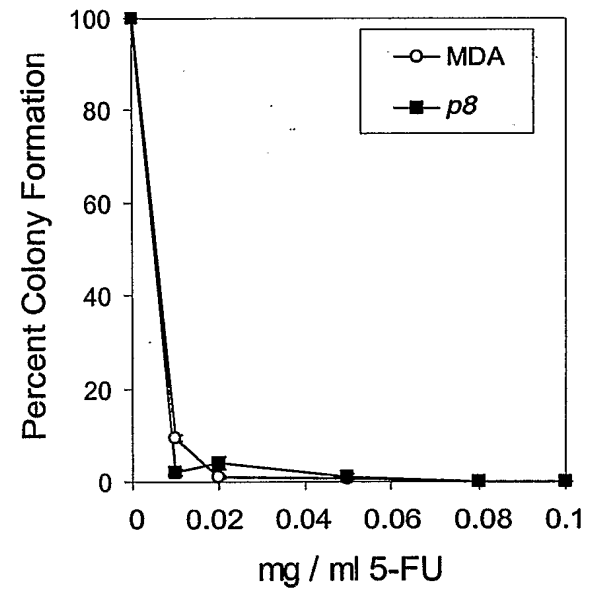
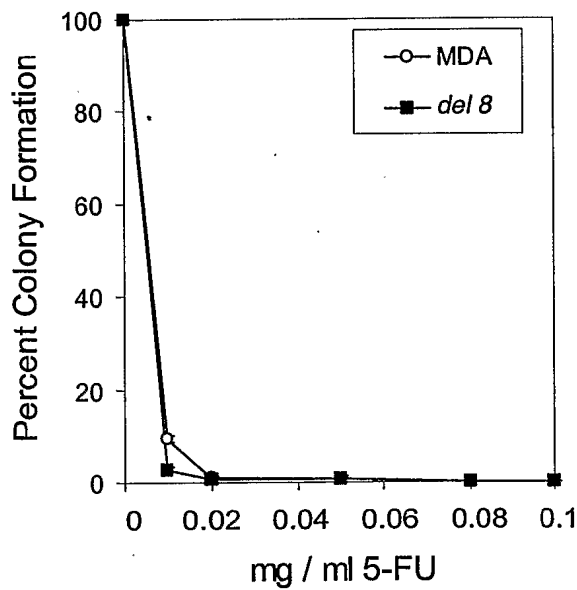
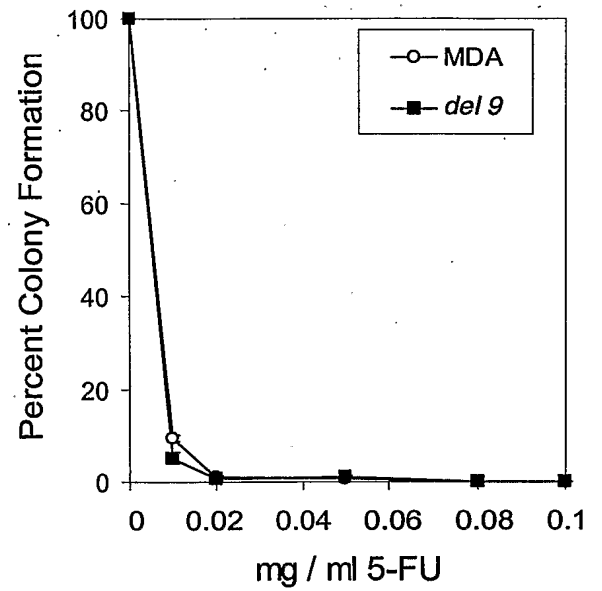
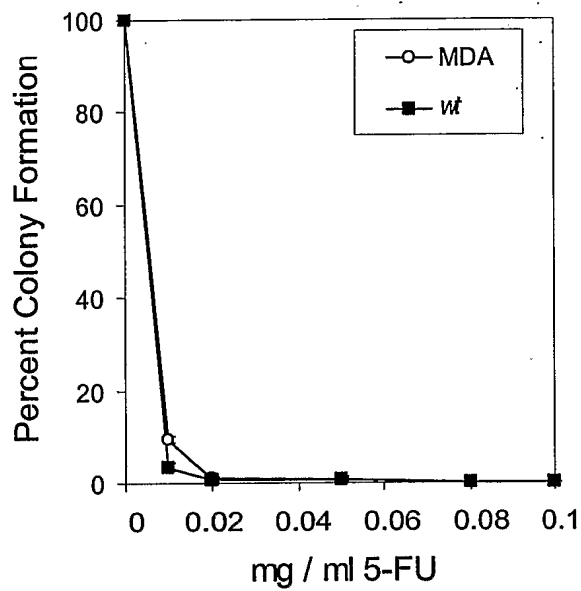
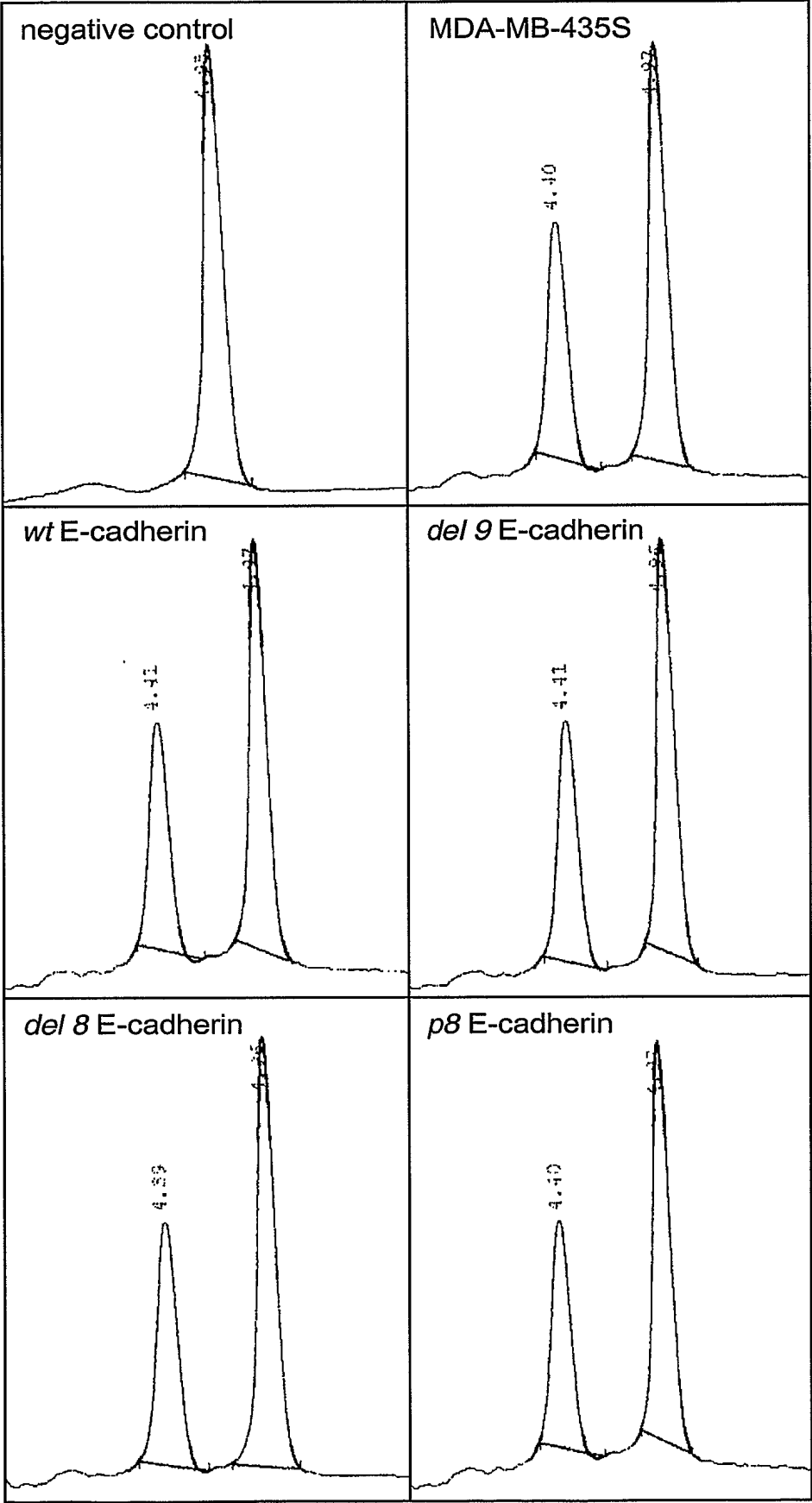
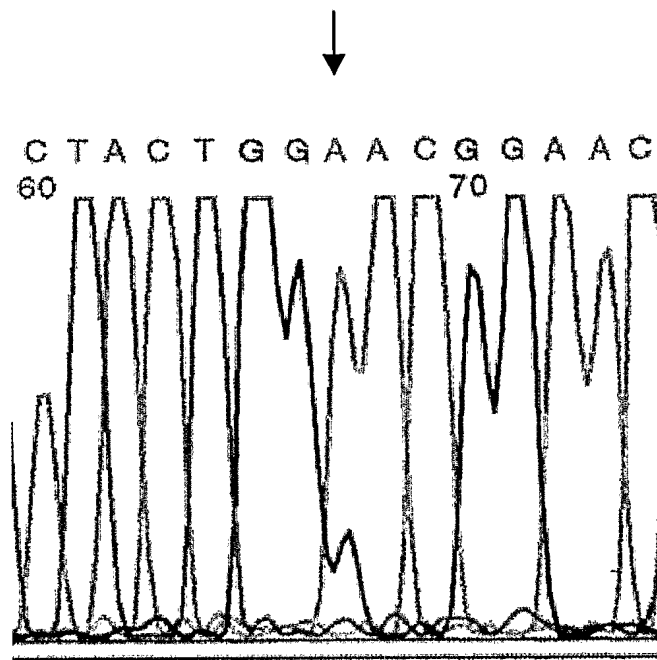
**B**

Figure 12 A



**Figure 12 B**

wild-type sequence: GGA  
mutant sequence: GAA



Figure 13



Figure 14

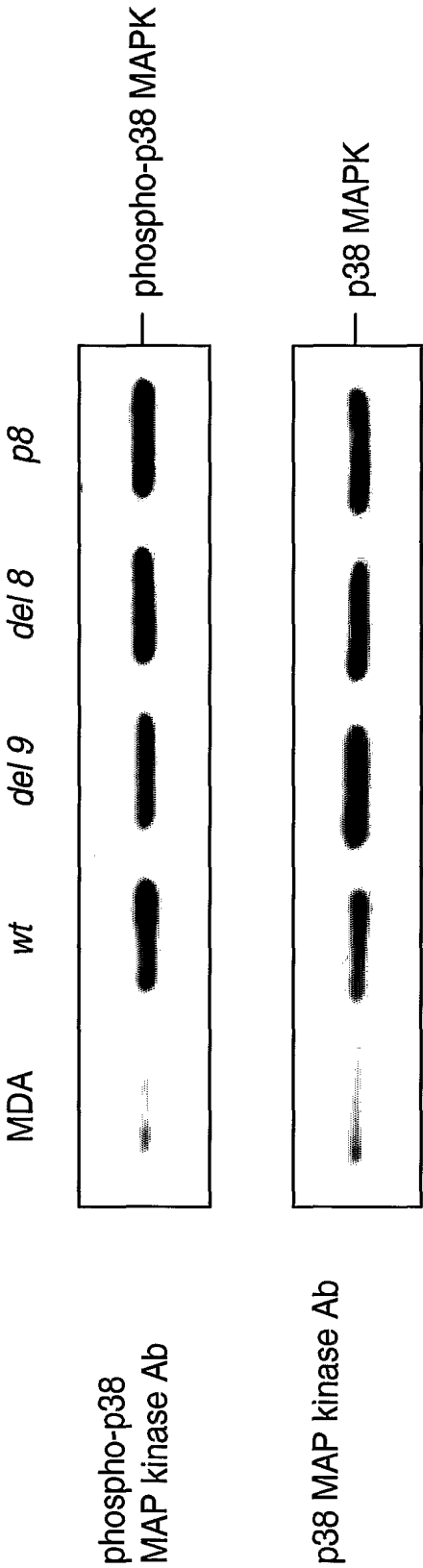


Figure 15

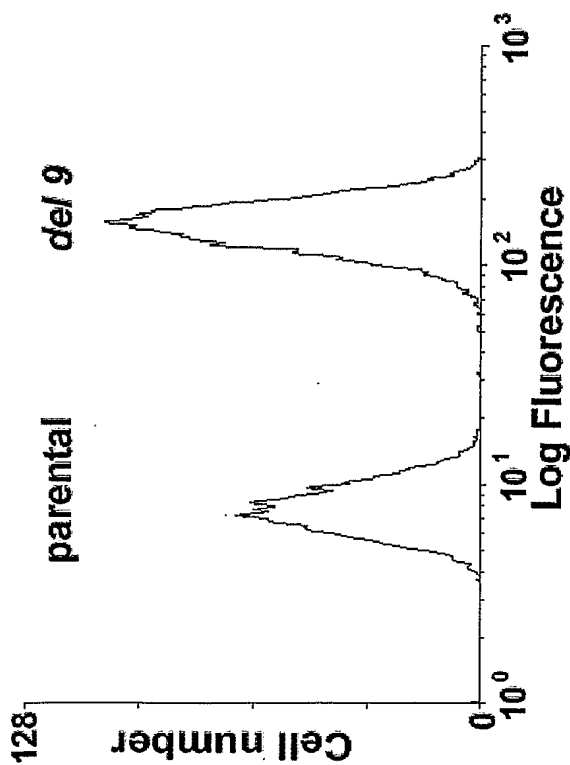
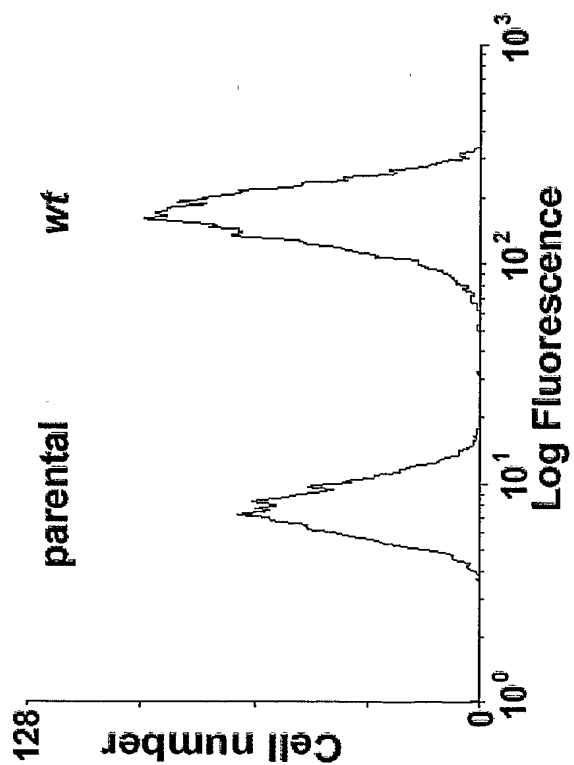
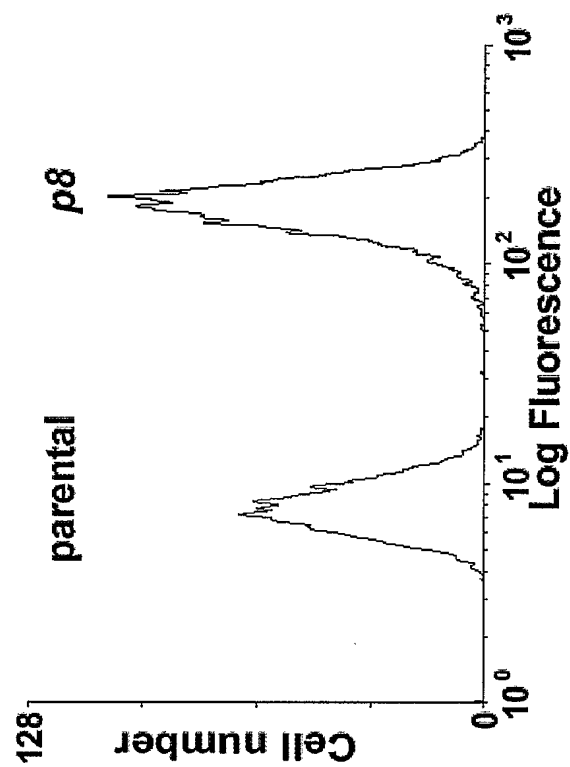
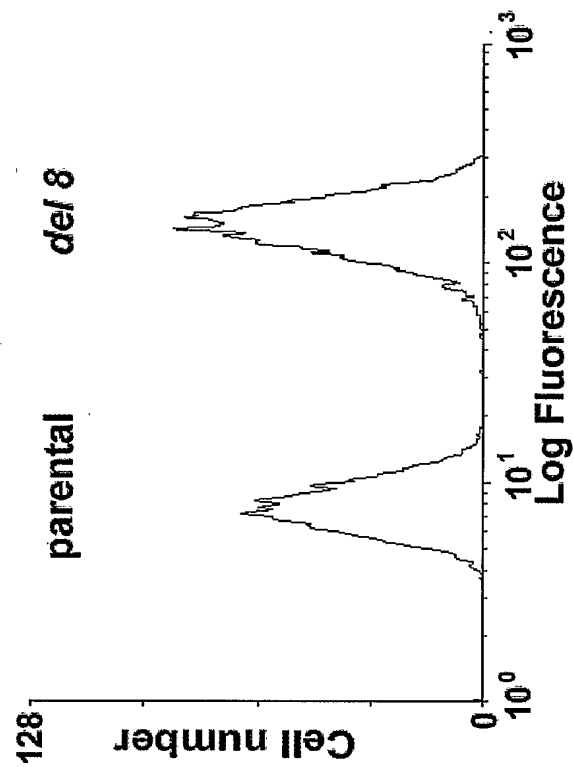


Figure 16 A

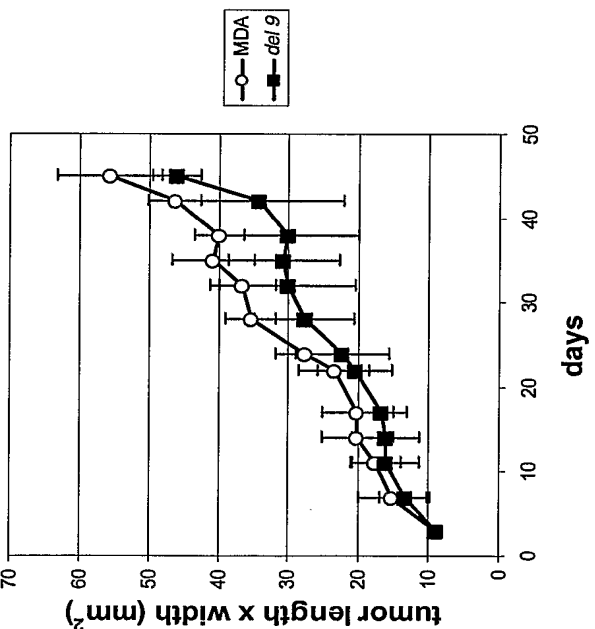
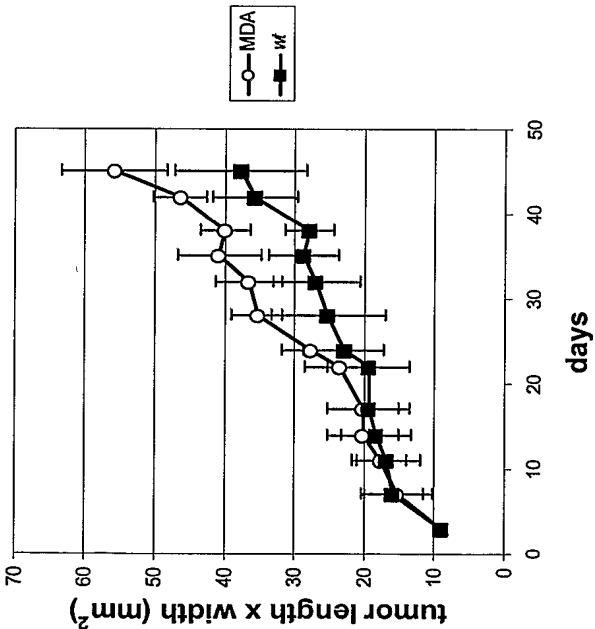
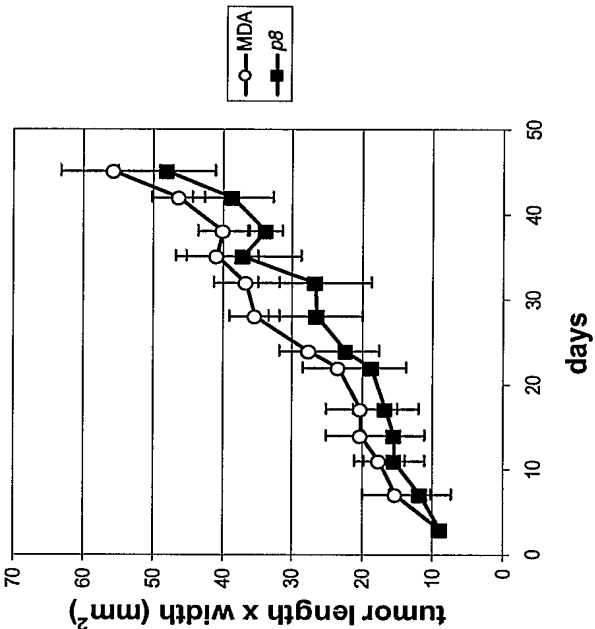
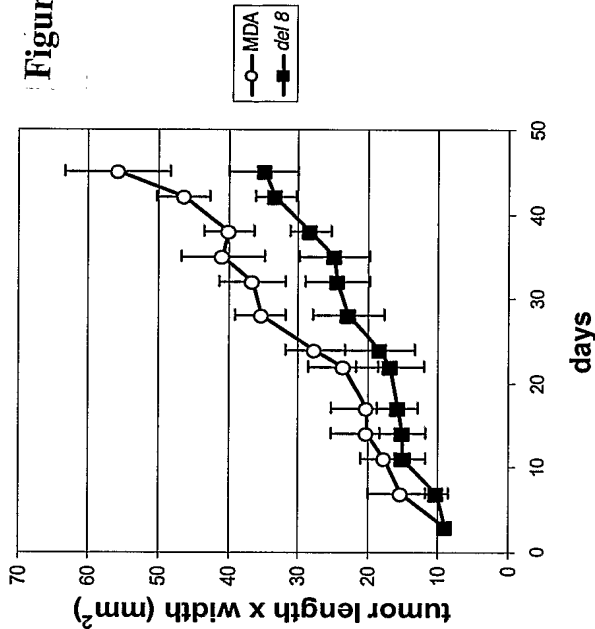


Figure 16 B

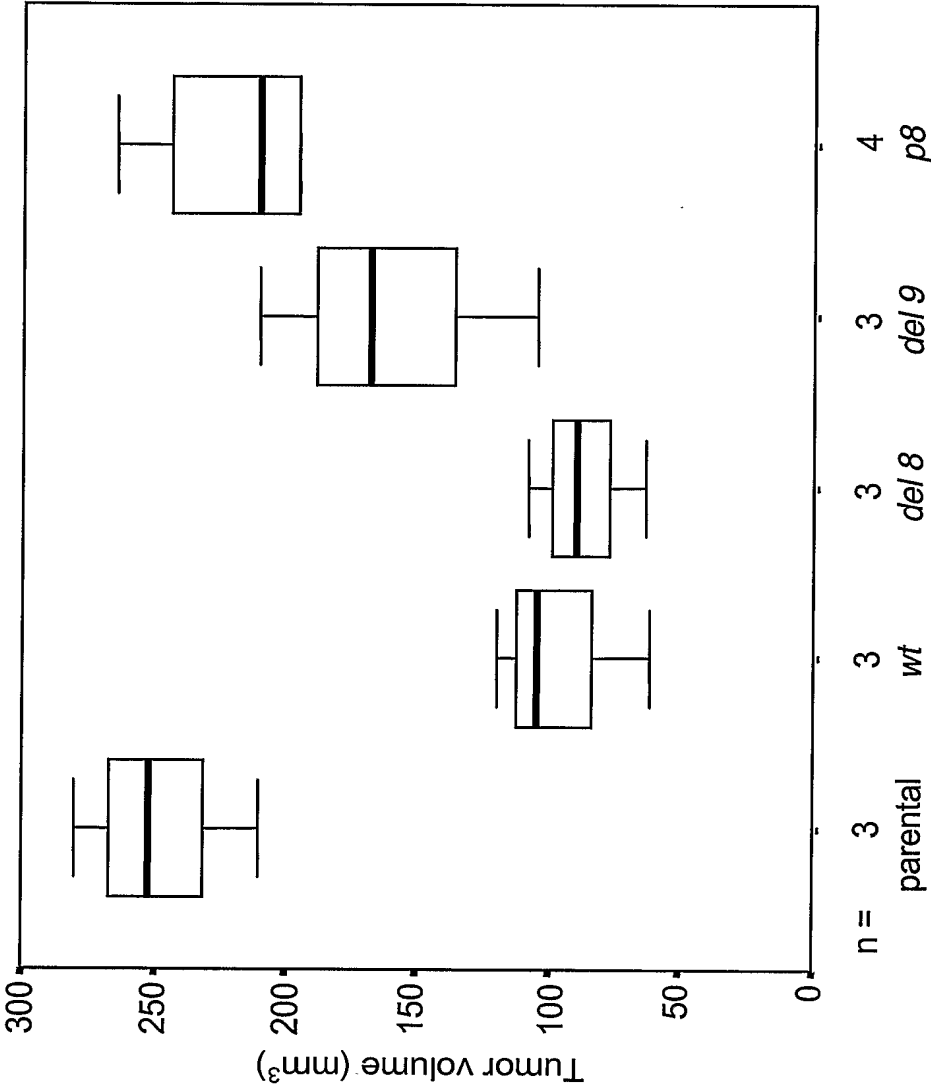
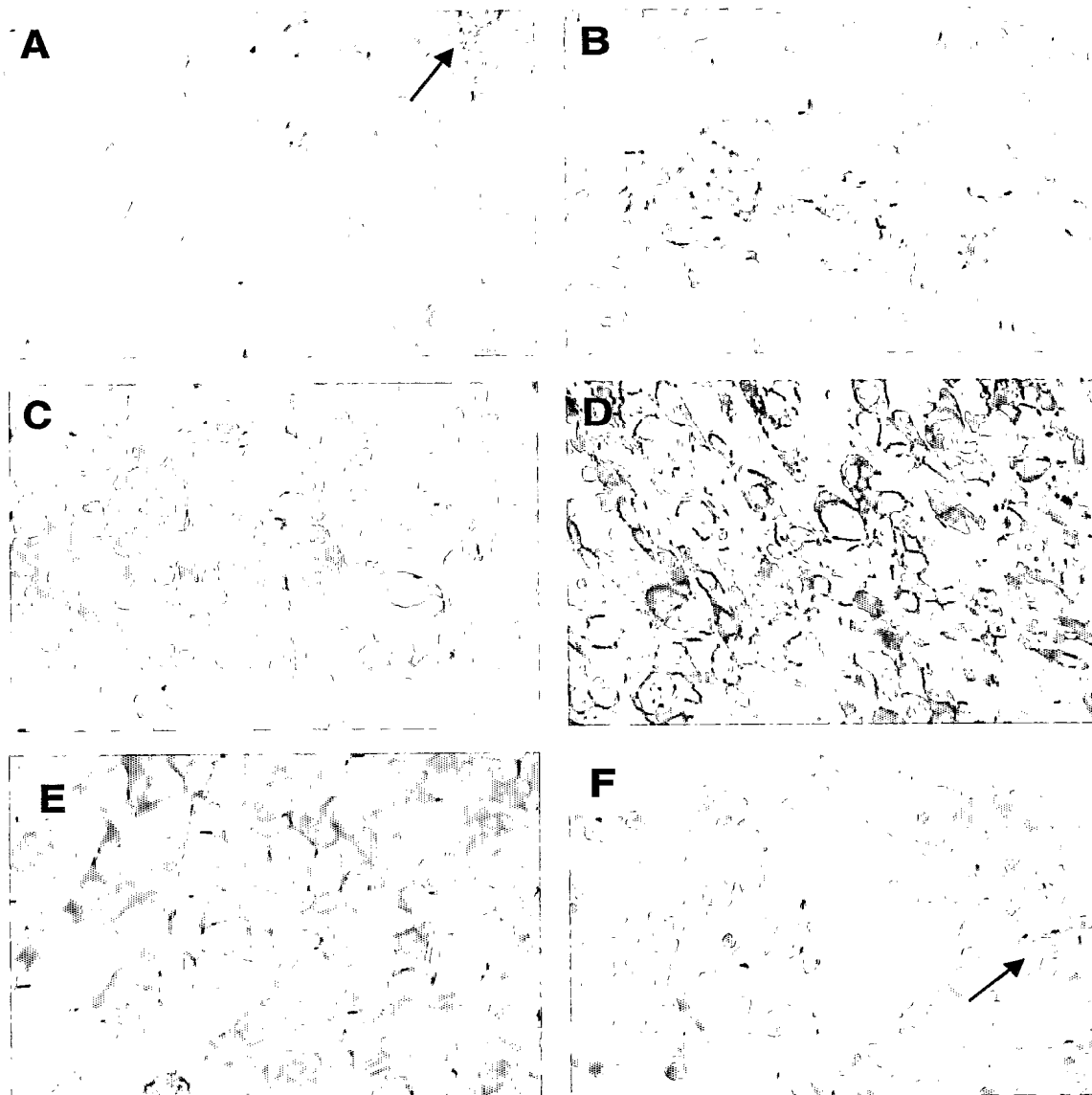


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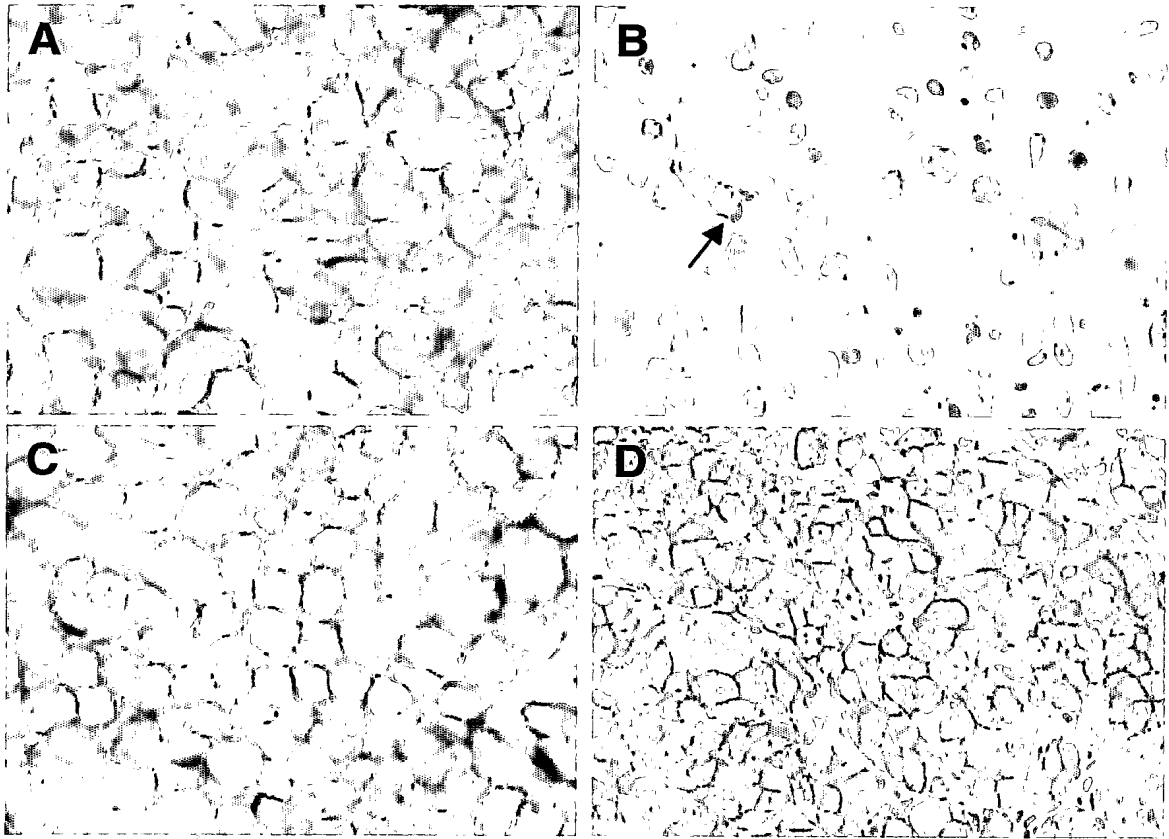
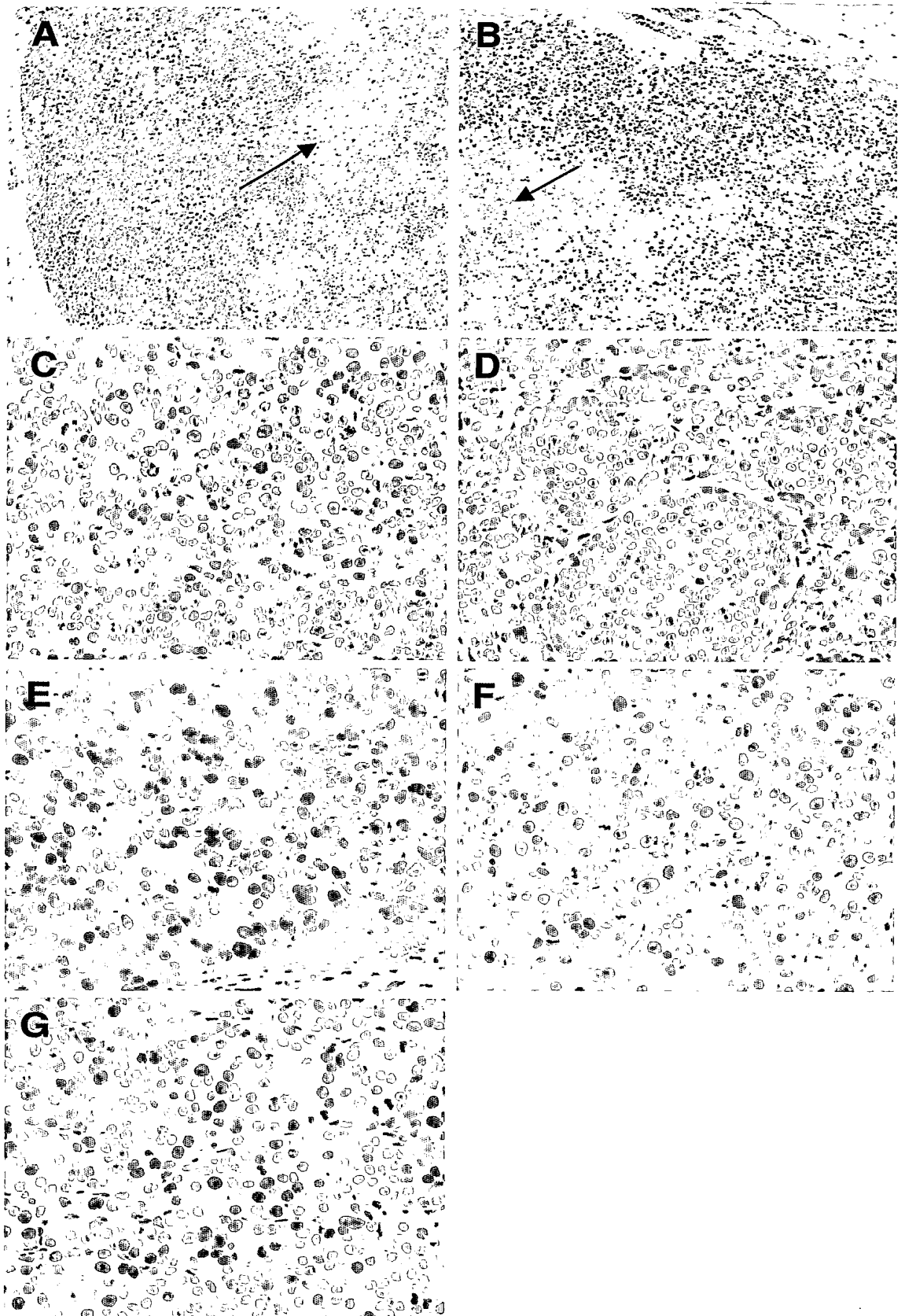
**Figure 18**

Figure 19





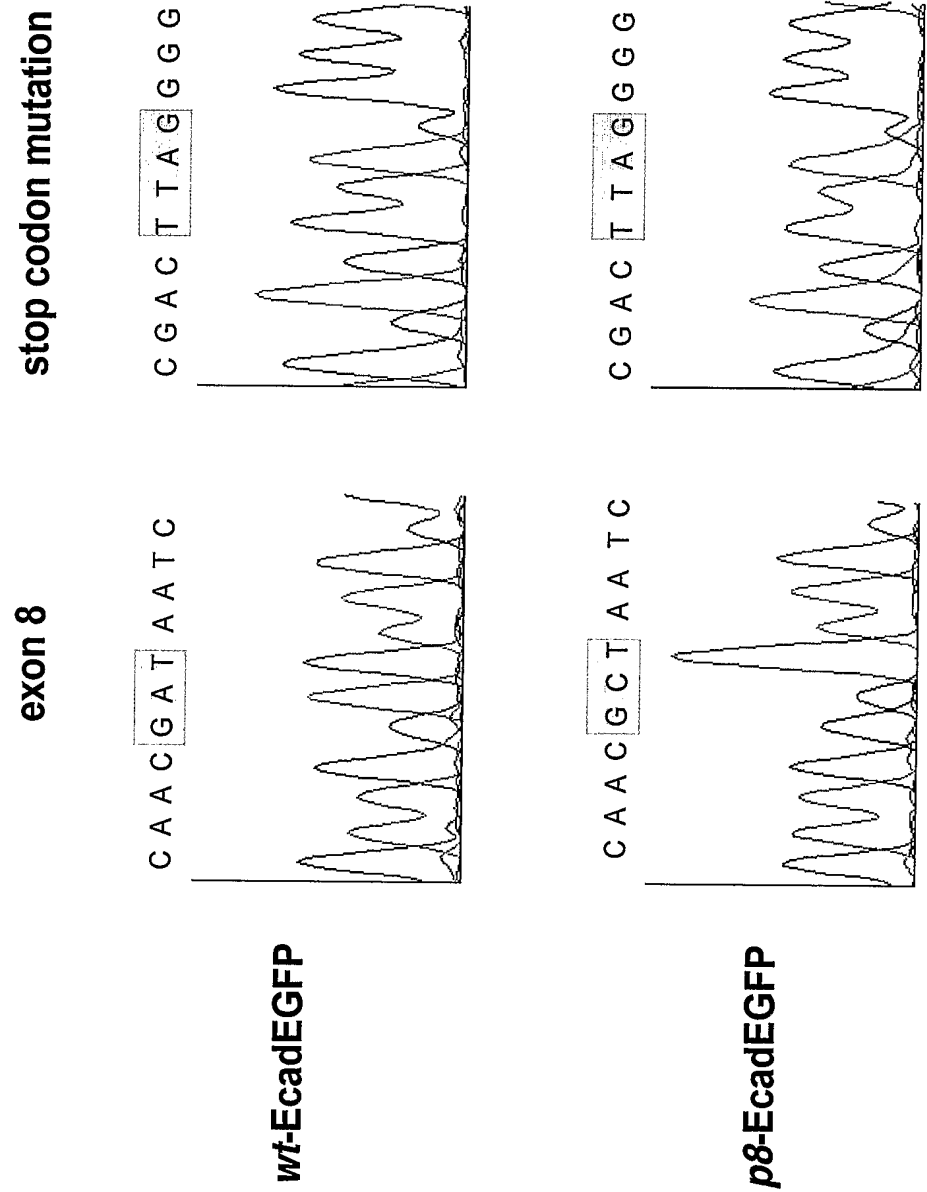
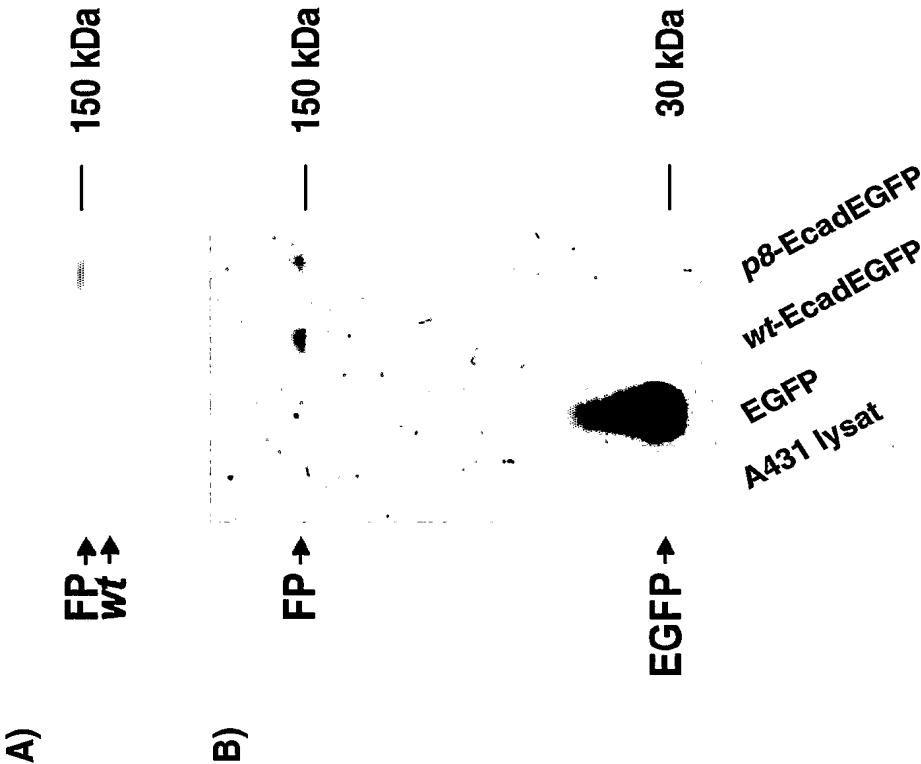


Figure 20

Figure 21



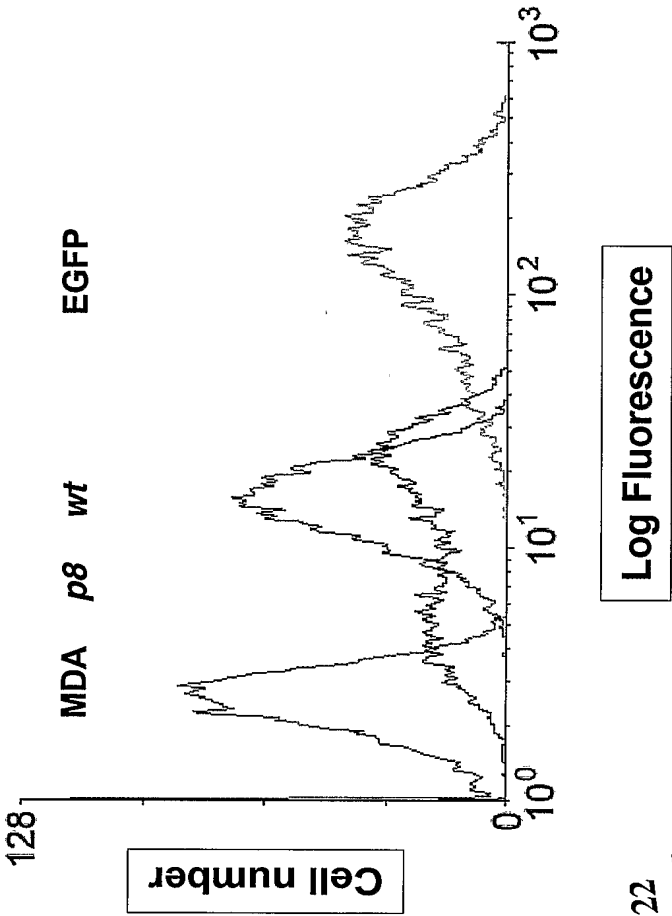


Figure 22

Figure 23

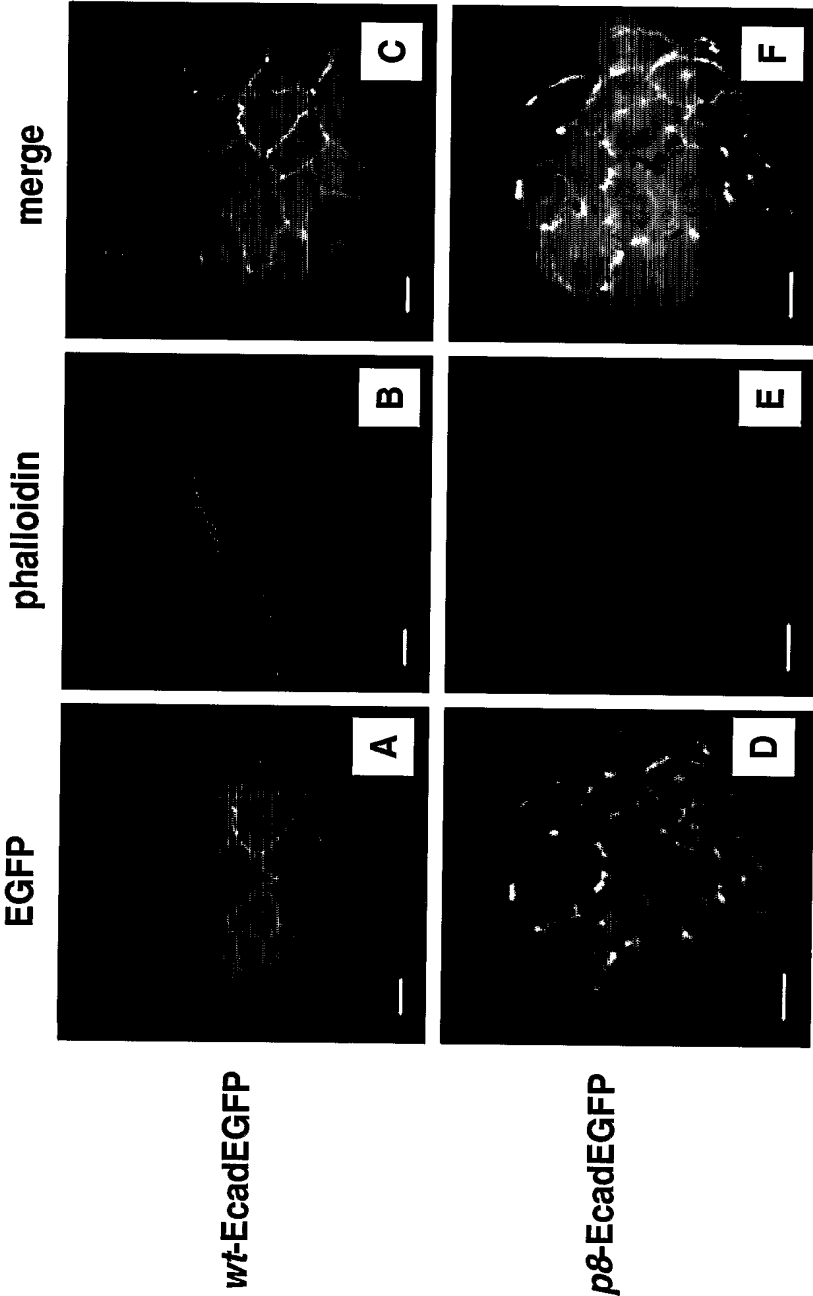


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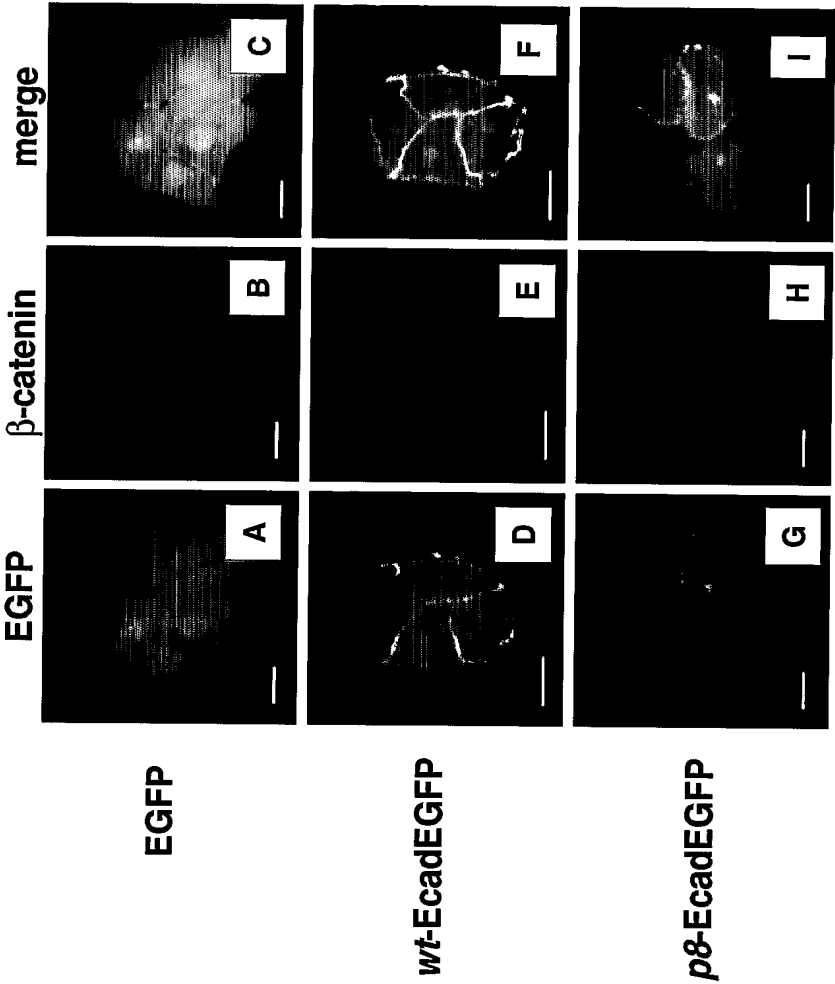


Figure 25

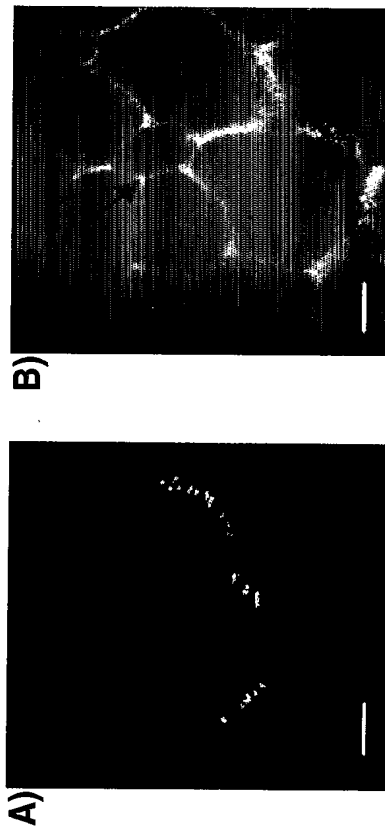
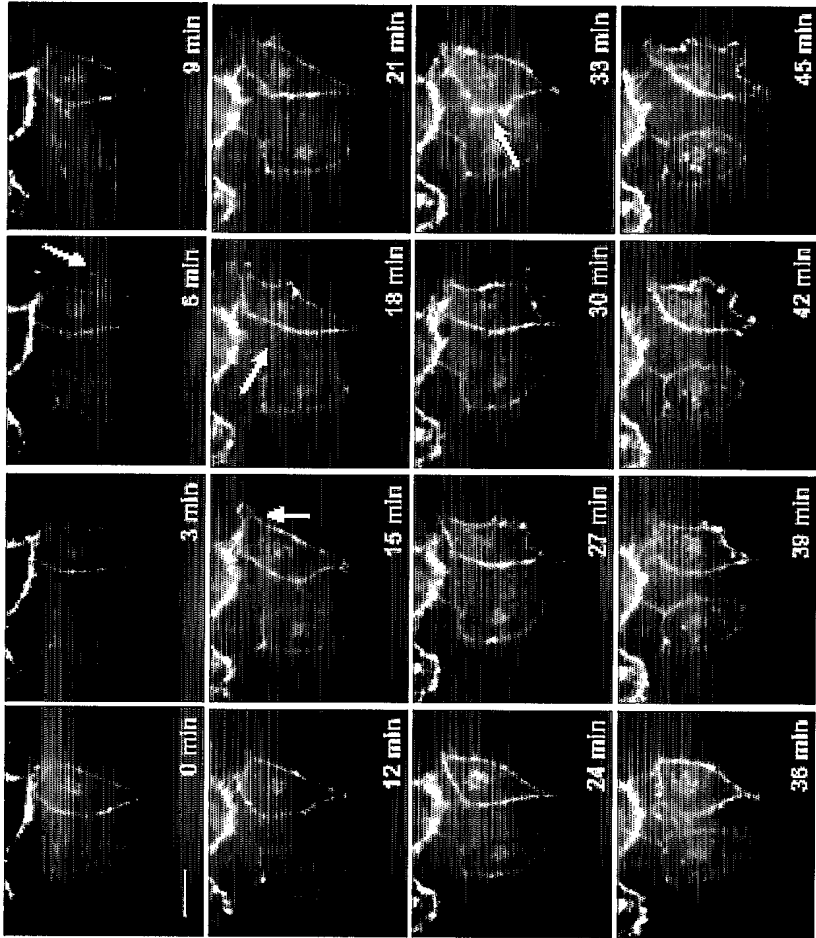


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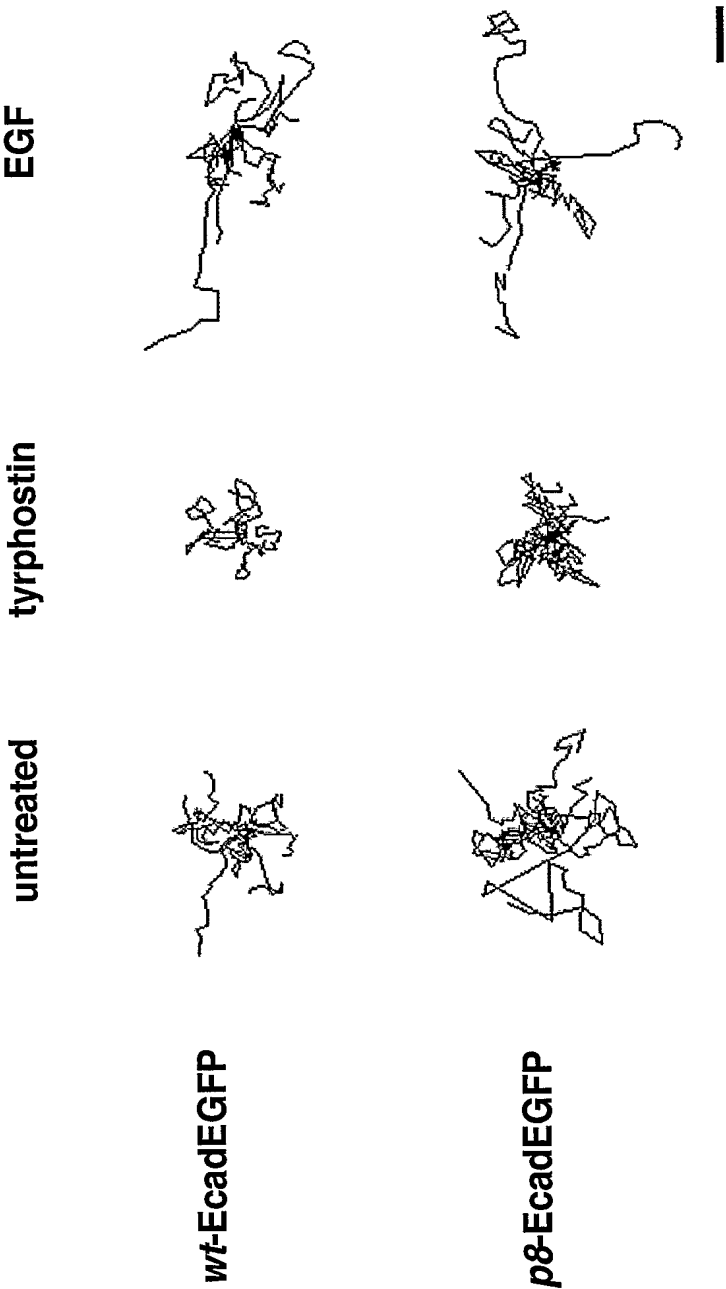


Figure 27



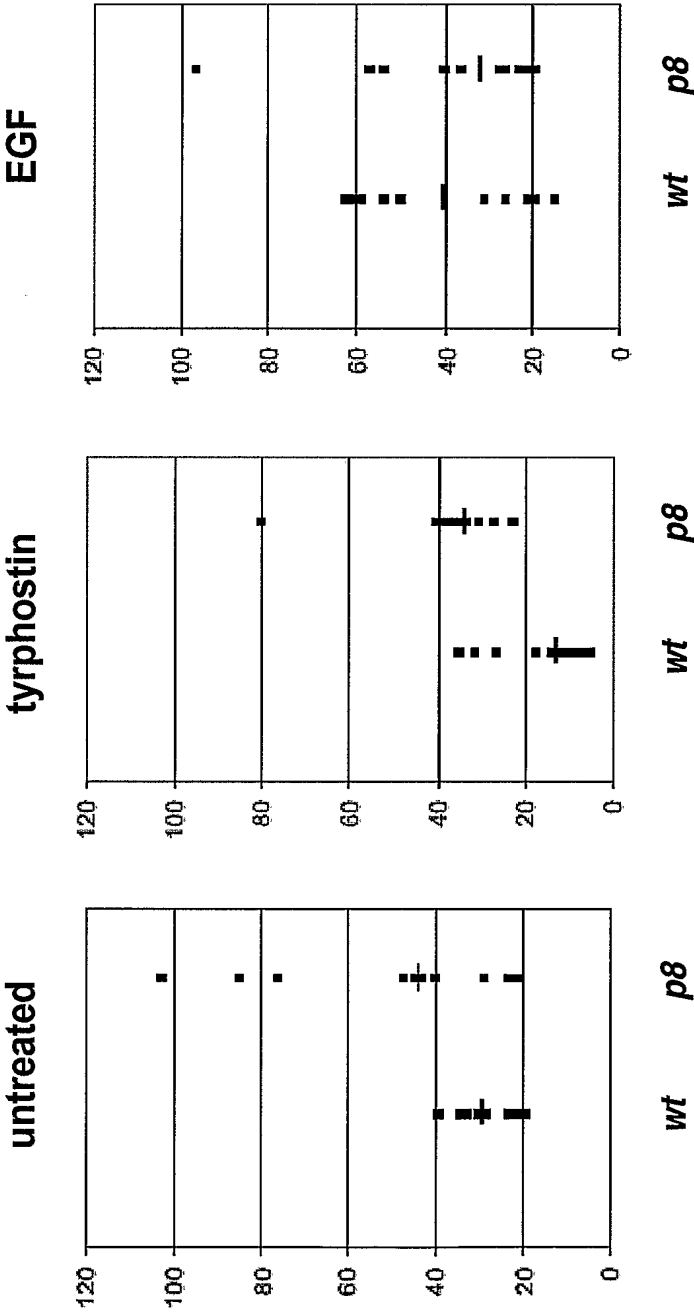


Figure 28

Figure 29

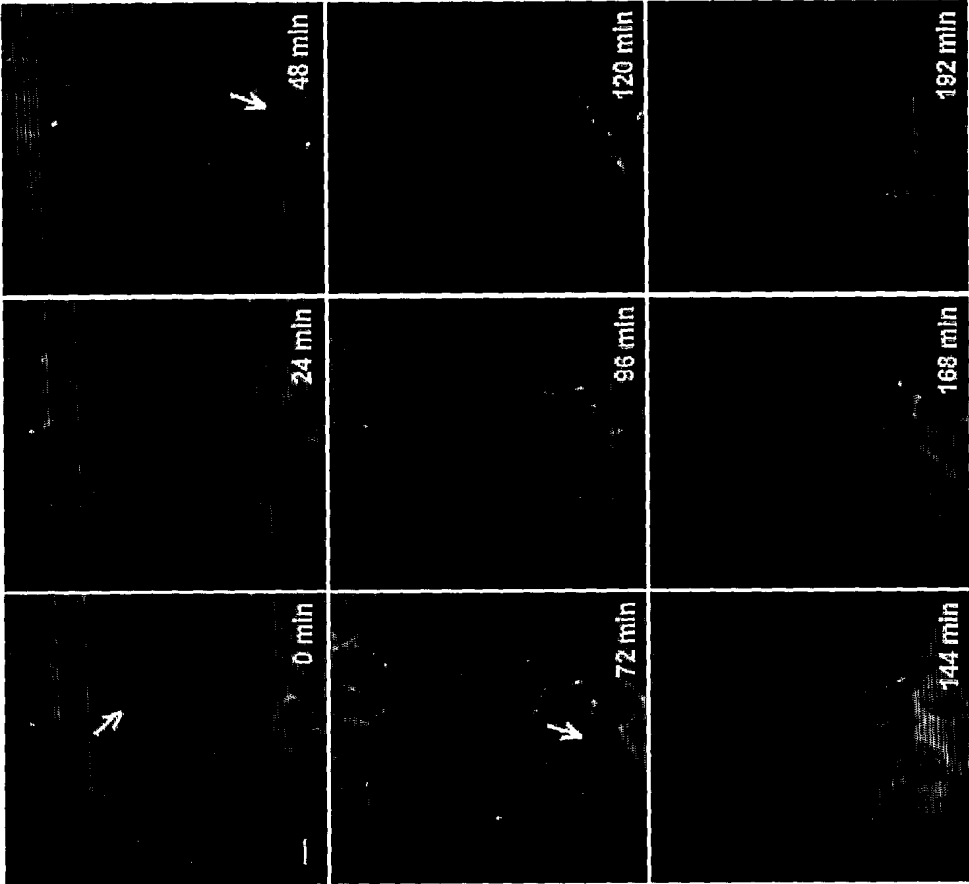


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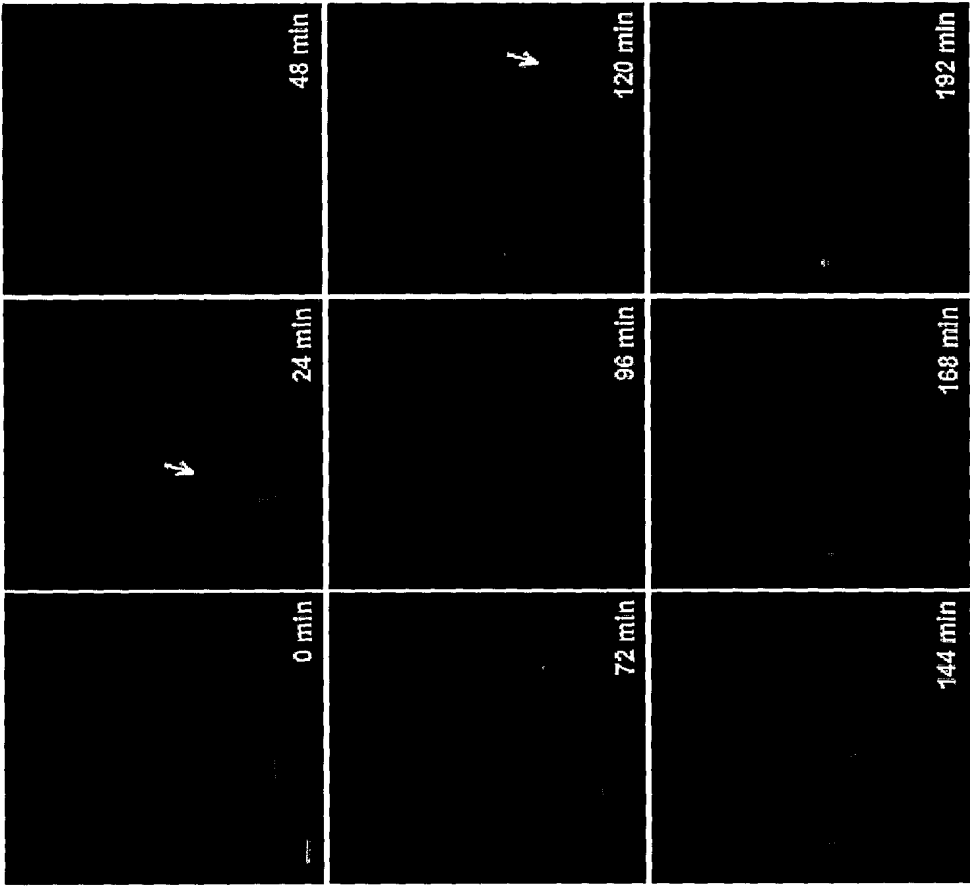


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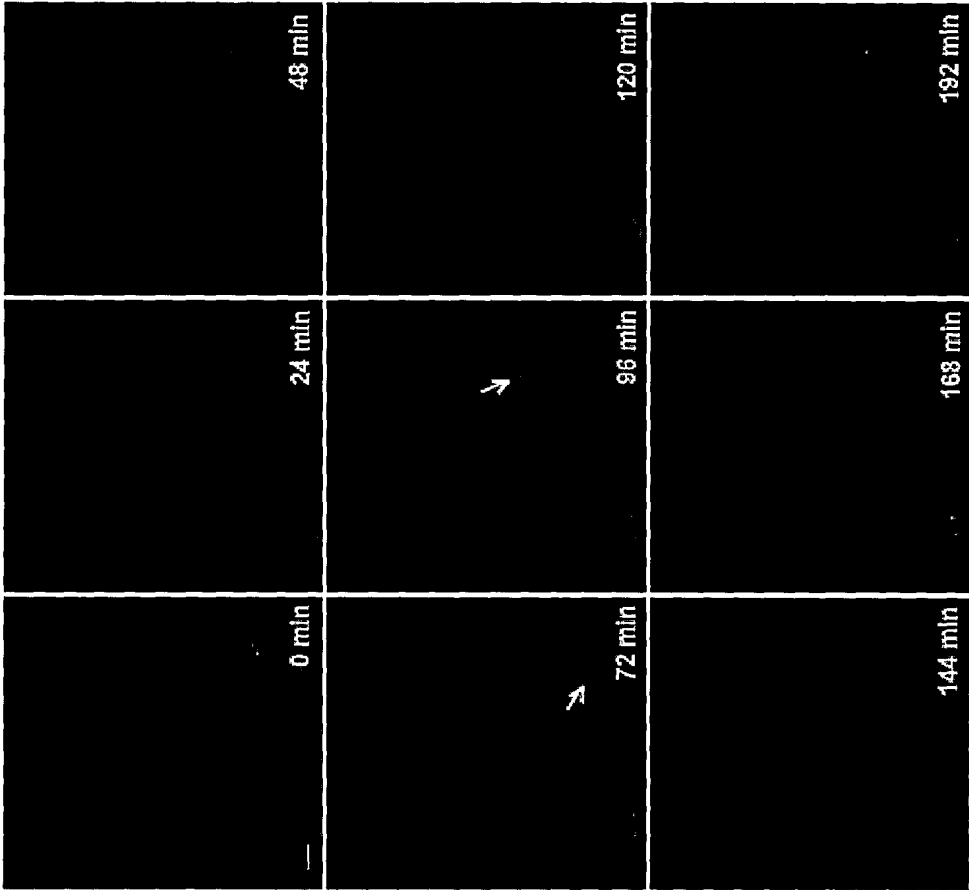


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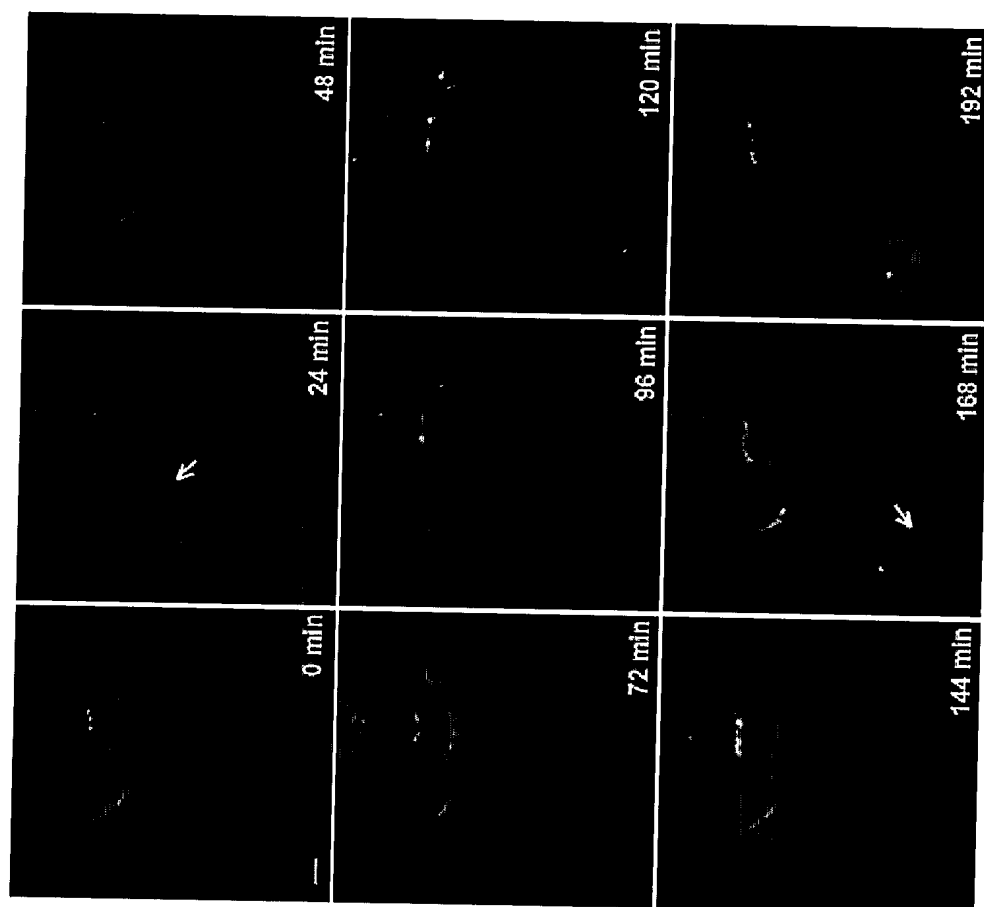


Figure 33

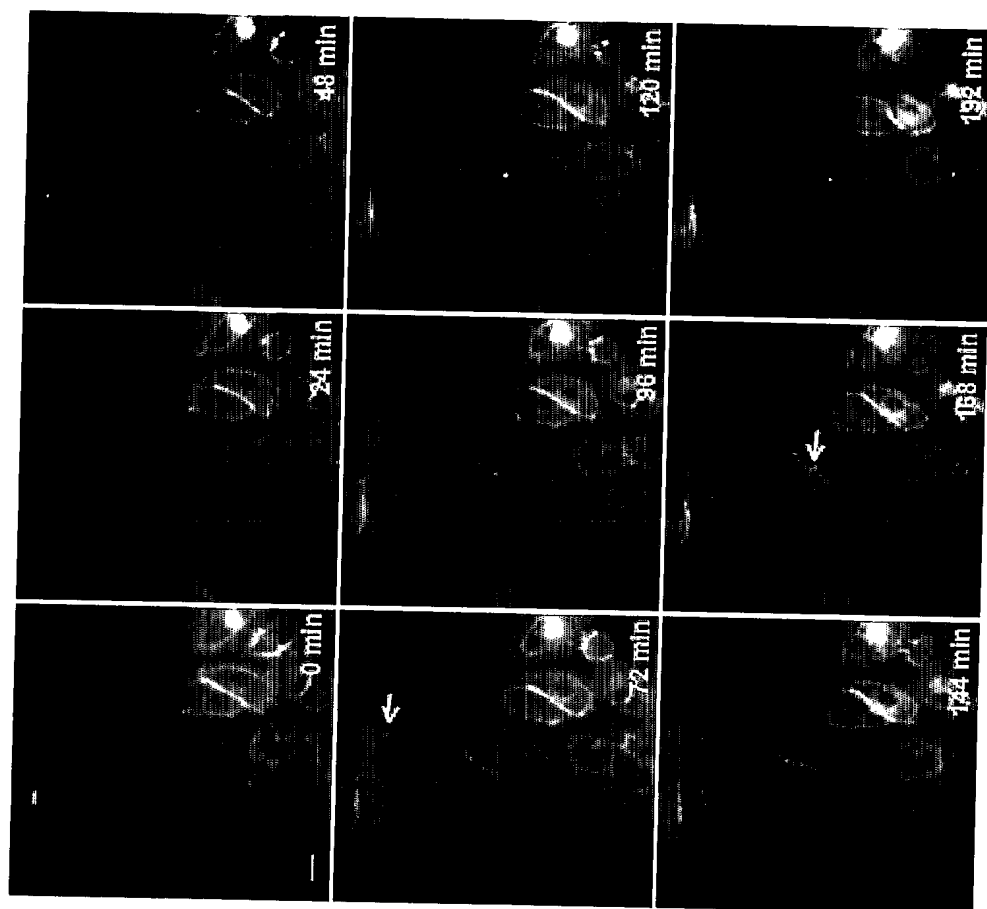
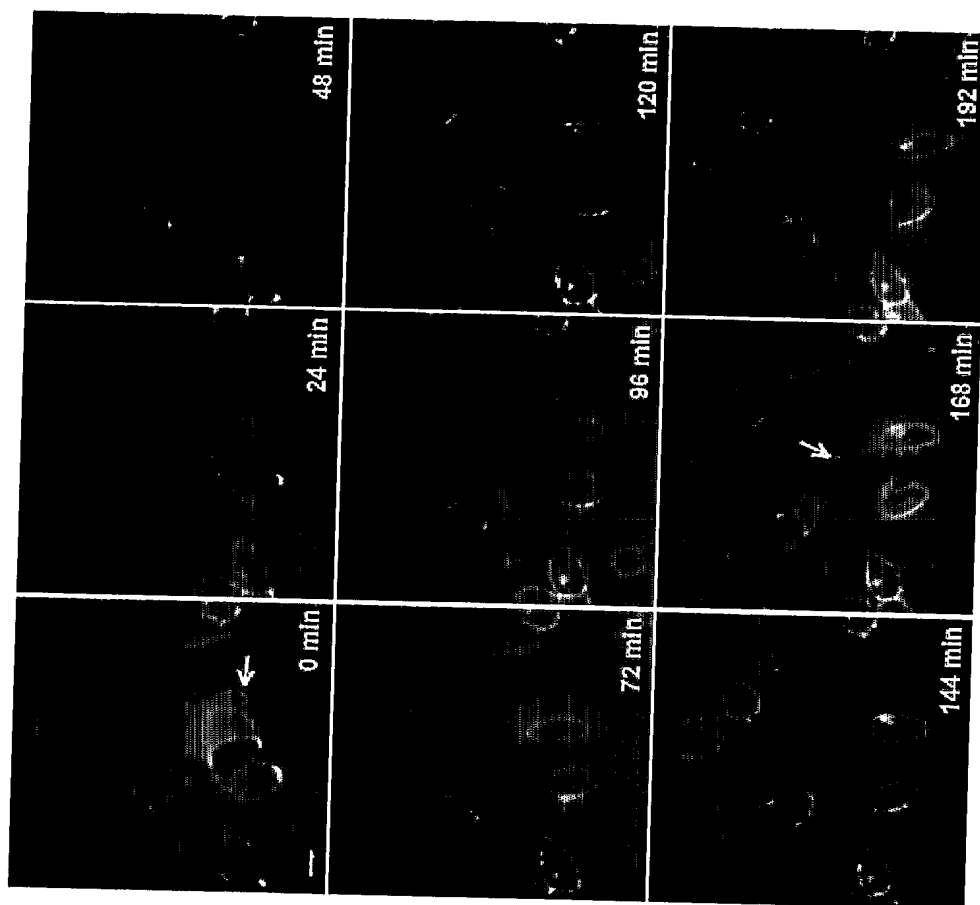


Figure 34



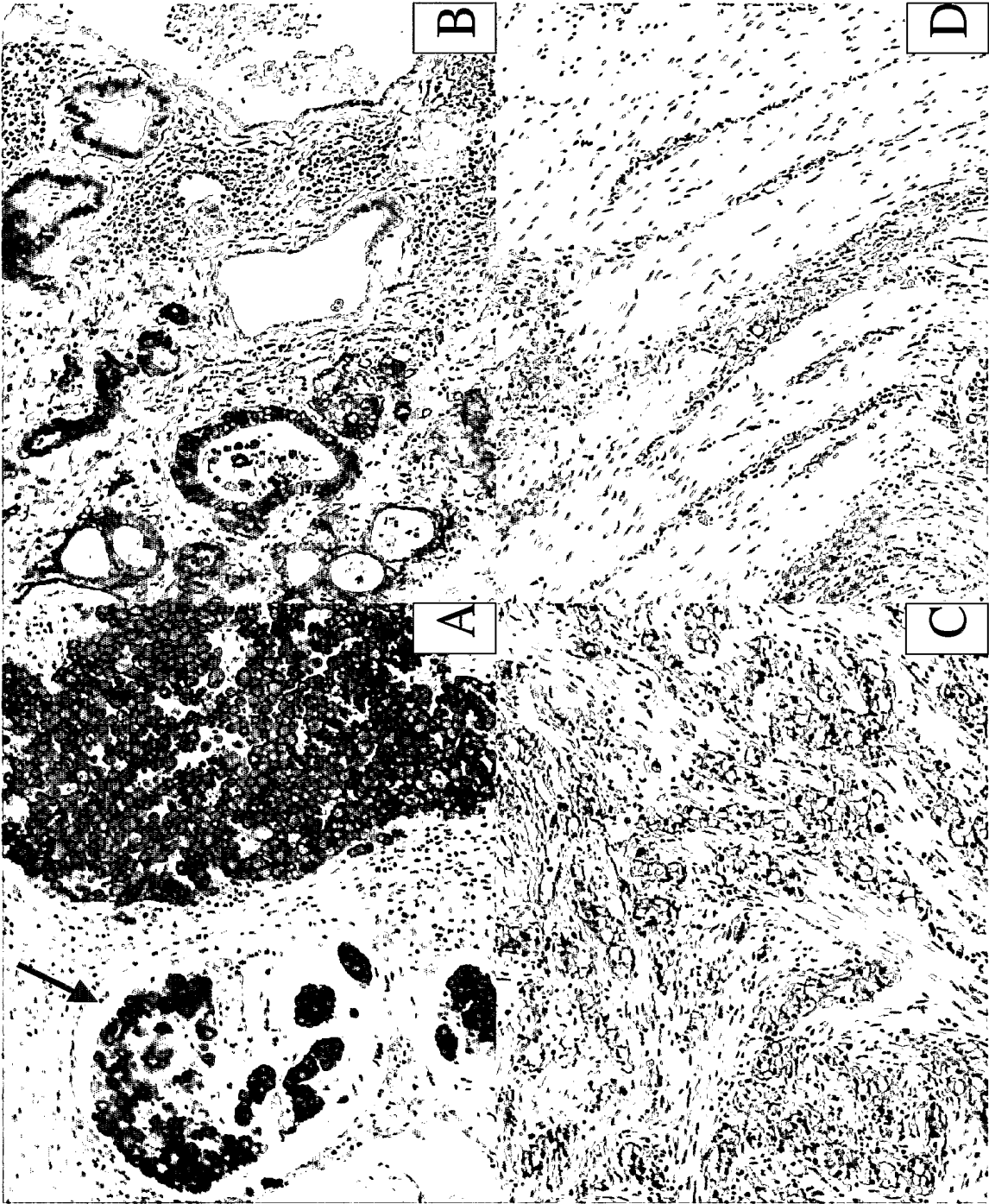
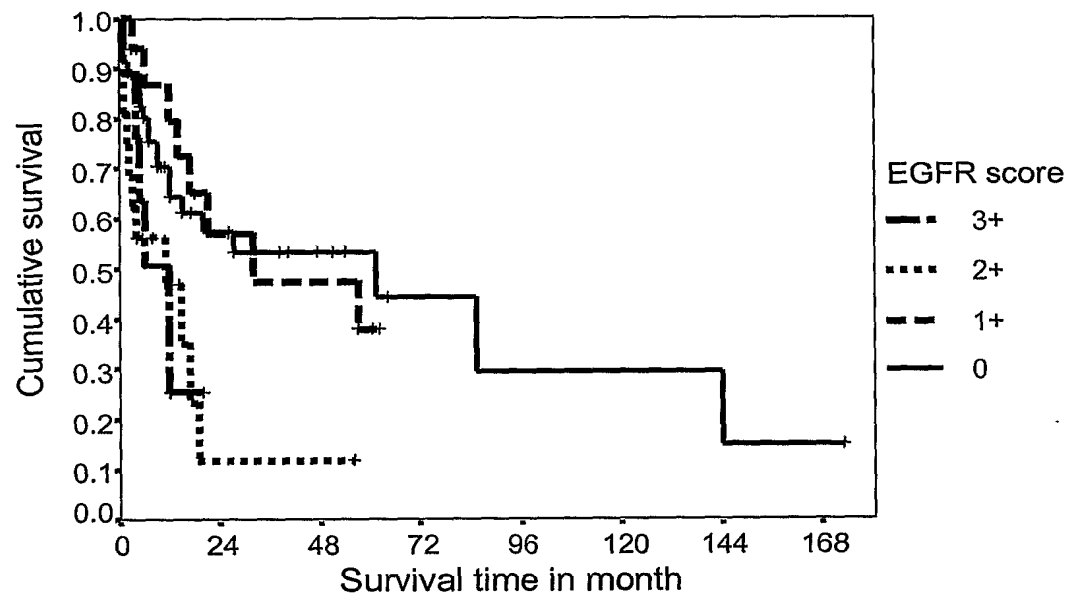


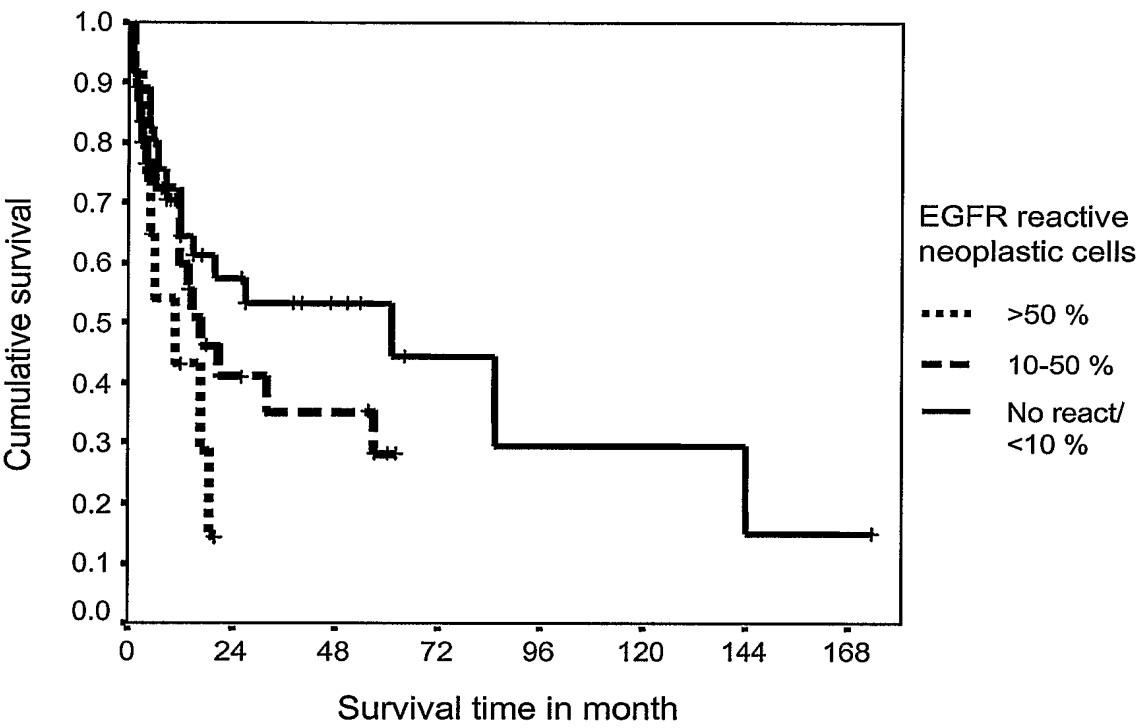


Figure 36



	Log Rank	
Global	0.0083	s
Score 0 vs 1+	0.9906	ns
Score 0 vs 2+	0.0070	s
Score 0 vs 3+	0.0552	ns
Score 1+ vs 2+	0.0088	s
Score 1+ vs 3+	0.0195	s
Score 2+ vs 3+	0.8424	ns
Score 0/1+ vs 2+/3+	0.0006	s

Figure 37



Global                      Log Rank                      0.0688                      ns

Figure 38

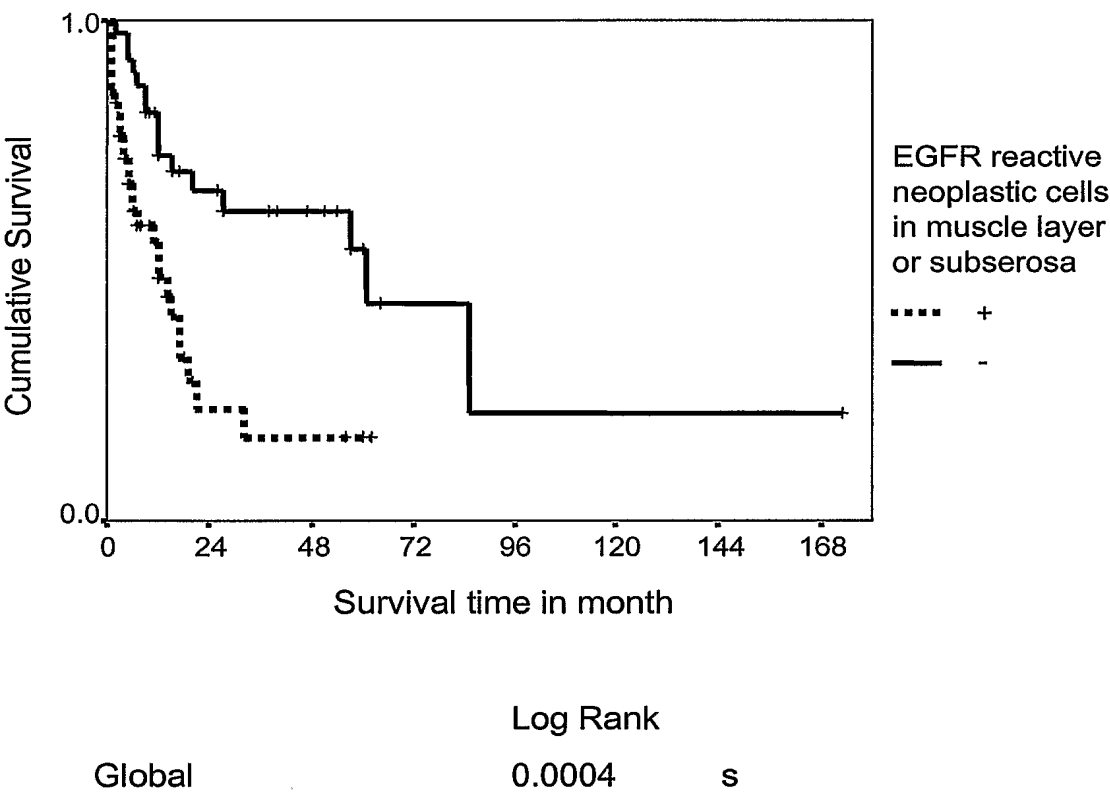
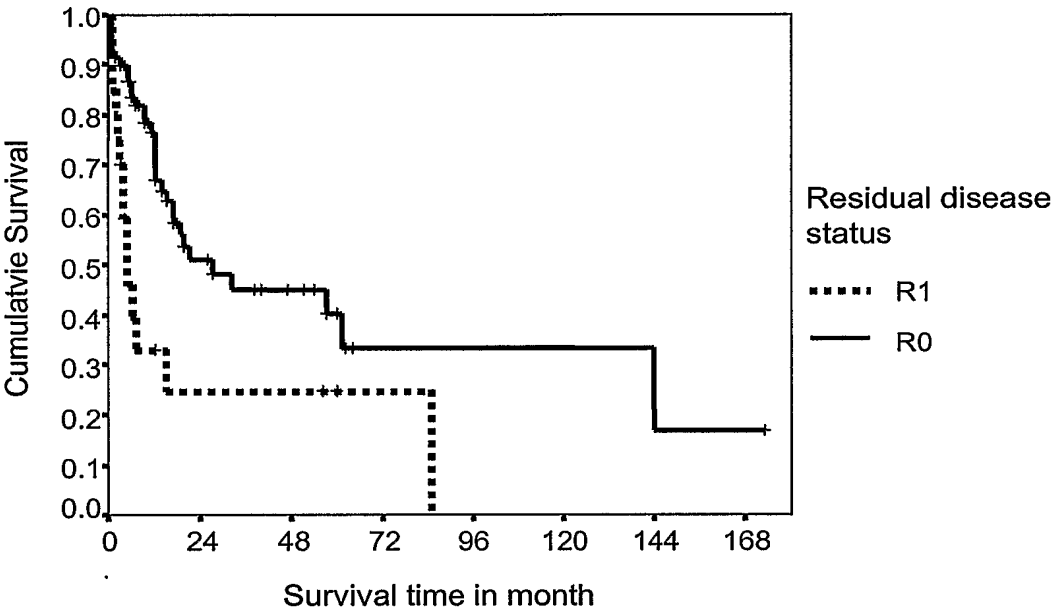


Figure 39



Global                      Log Rank                      0.0028                      s

Figure 40

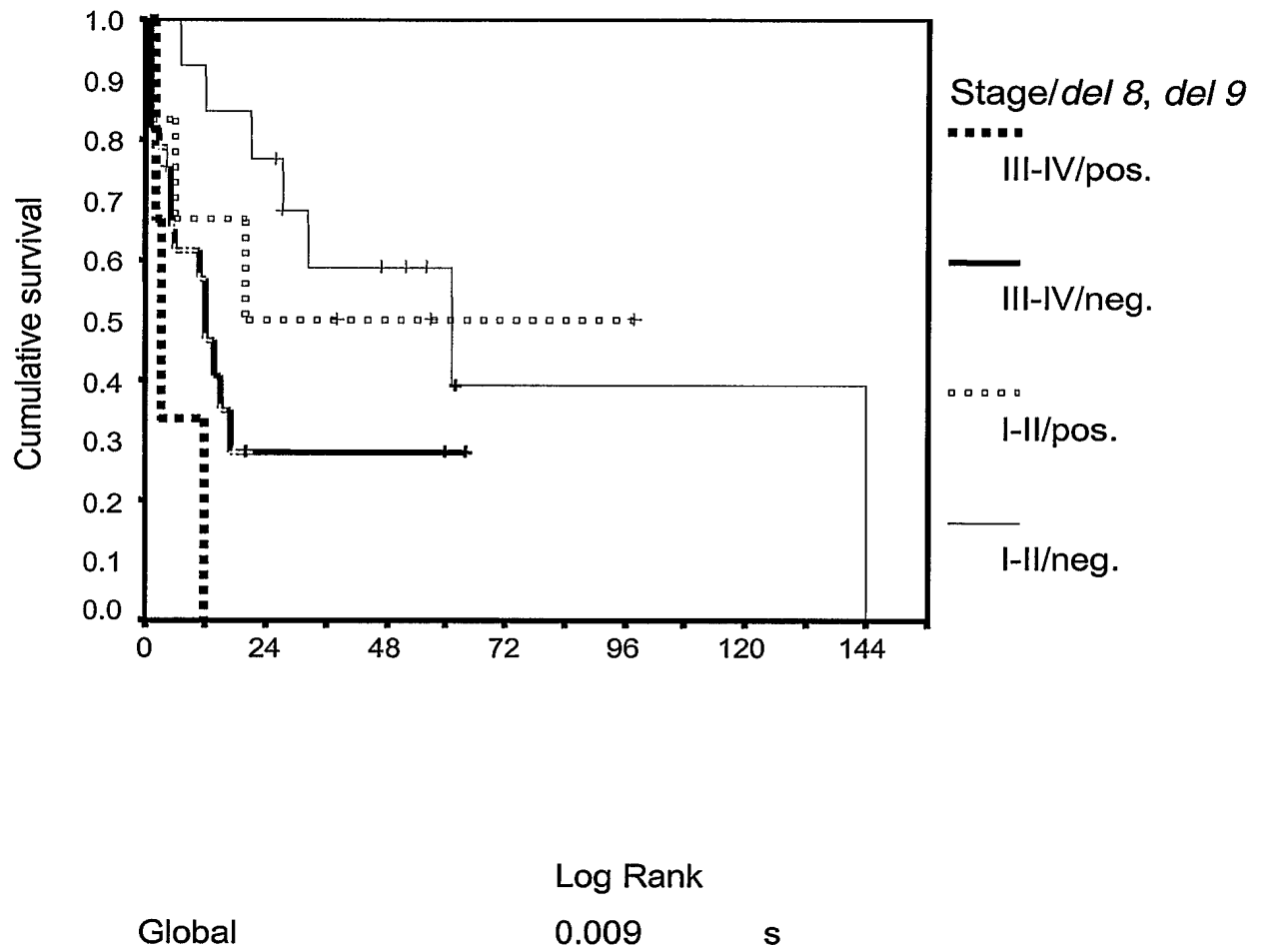
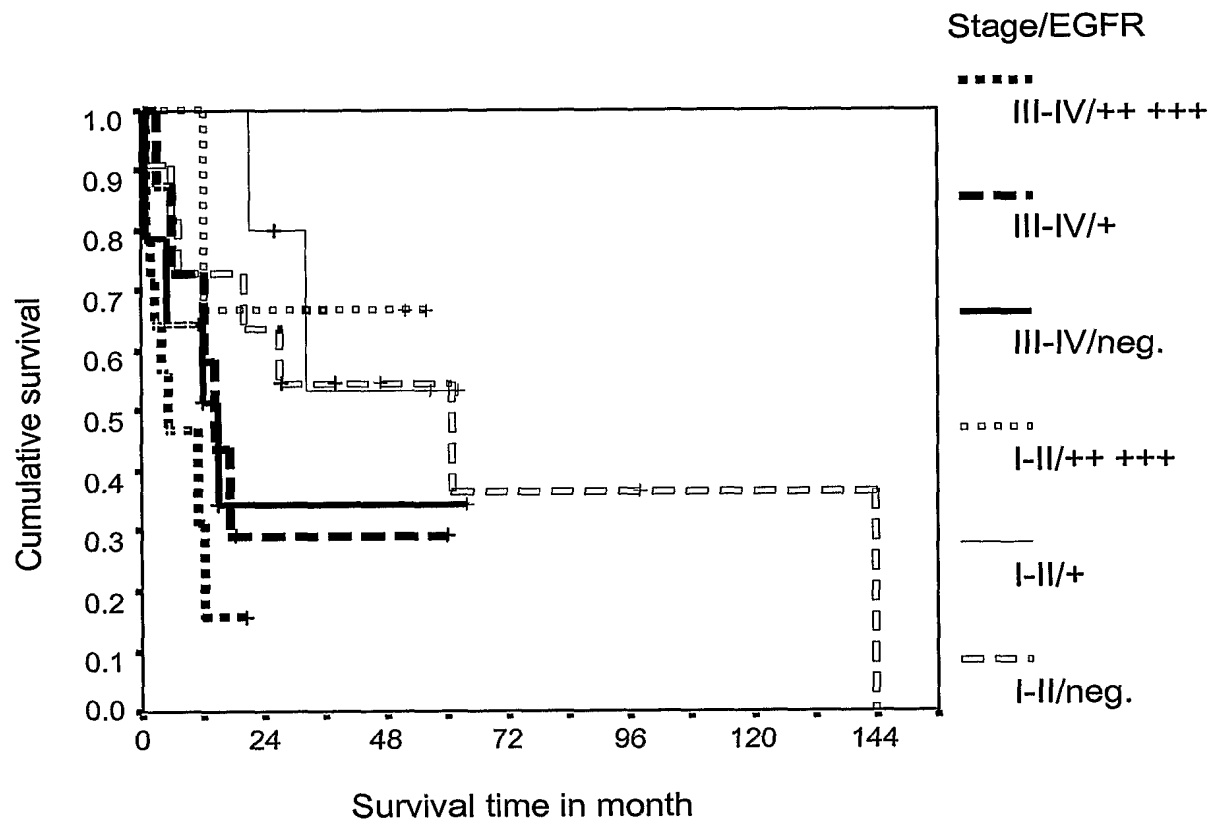


Figure 41



Log Rank  
Global 0.0326 s

(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
27 November 2003 (27.11.2003)

PCT

(10) International Publication Number  
**WO 2003/097086 A3**

(51) International Patent Classification<sup>7</sup>: **C07K 14/475**,  
14/71

(74) Agent: **VOSSIUS & PARTNER**; Siebertstrasse 4, 81675  
Munich (DE).

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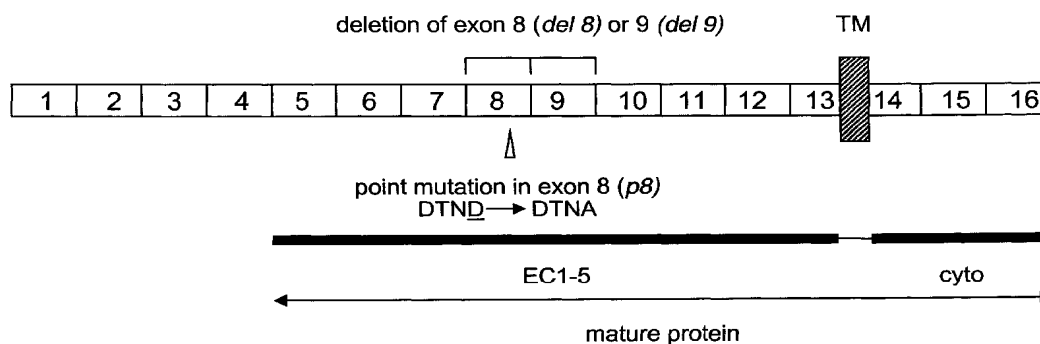
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ning of each regular issue of the PCT Gazette.*

(54) Title: EGF RECEPTOR ANTAGONISTS IN THE TREATMENT OF GASTRIC CANCER



(57) Abstract: The present invention relates to a use of (an) EGF receptor antagonist(s)/inhibitor(s) for the preparation of a pharmaceutical composition for the prevention, amelioration or treatment of gastric carcinomas, preferably for the prevention, amelioration or treatment of diffuse gastric carcinomas. Furthermore, the invention provides for a method for treating or for preventing gastric carcinomas, in particular diffuse gastric carcinomas comprising the administration of at least one EGF receptor antagonist/inhibitor to a subject in need of such a treatment or prevention.

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/05057

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/475 C07K14/71

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, MEDLINE, WPI Data, PAJ, BIOSIS, EMBASE, SCISEARCH, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FUCHS MARGIT ET AL: "Motility enhancement by tumor-derived mutant E-cadherin is sensitive to treatment with epidermal growth factor receptor and phosphatidylinositol 3-kinase inhibitors." EXPERIMENTAL CELL RESEARCH. UNITED STATES 10 JUN 2002, vol. 276, no. 2, 10 June 2002 (2002-06-10), pages 129-141, XP002265554 ISSN: 0014-4827 the whole document --- -/--	1-12

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
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- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*Z\* document member of the same patent family

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## INTERNATIONAL SEARCH REPORT

International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>ANDERSON N G ET AL: "EFFECTS OF ZD 1839 (IRESSA), A NOVEL EGF RECEPTOR TYROSINE KINASE INHIBITOR, ON BRAST CANCER CELL PROLIFERATION AND INVASIVENESS" BREAST CANCER RESEARCH AND TREATMENT, NIJHOFF, BOSTON, US, vol. 64, no. 1, November 2000 (2000-11), page 32 XP001062478 ISSN: 0167-6806 the whole document</p>	1-12
Y	<p>WO 99 62955 A (EPA VIDANAGAMAGE CHANDANA ;GARRETT THOMAS PETER JOHN (AU); MCKERN) 9 December 1999 (1999-12-09) the whole document</p>	1-12
Y	<p>EP 0 821 060 A (GSF FORSCHUNGSZENTRUM UMWELT) 28 January 1998 (1998-01-28) the whole document</p>	1-12
Y	<p>WO 99 20168 A (GUILFORD PARRY JOHN ;TE WHETU WHANAU TRUST LIMITED (NZ); UNIV OTAG) 29 April 1999 (1999-04-29) the whole document</p>	1-12
Y	<p>ELLIS A G ET AL: "High-performance liquid chromatographic analysis of the tyrphostin AG1478, a specific inhibitor of the epidermal growth factor receptor tyrosine kinase, in mouse plasma" JOURNAL OF CHROMATOGRAPHY. BIOMEDICAL APPLICATIONS, ELSEVIER, AMSTERDAM, NL, vol. 754, no. 1, 15 April 2001 (2001-04-15), pages 193-199, XP004232006 ISSN: 0378-4347 the whole document</p>	1-12
A	<p>ZOELEN VAN E J J ET AL: "RATIONAL DESIGN FOR THE DEVELOPMENT OF EPIDERMAL GROWTH FACTOR RECEPTOR ANTAGONISTS" PATHOLOGY RESEARCH AND PRACTICE, GUSTAV FISCHER, STUTTGART, DE, vol. 192, no. 7, July 1996 (1996-07), pages 761-767, XP000892672 ISSN: 0344-0338</p>	

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/EP 03/05057

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: —  
because they relate to subject matter not required to be searched by this Authority, namely:  
Rule 39.1(iv) PCT – Method for treatment of the human or animal body by therapy (Claims 9 and 12)
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 03/05057

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